THE PURIFICATION AND CHARACTERISATION OF HUMAN BRADYKININOGEN

by

George E. Mawer

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I INTRODUCTION

GLOSSARY

Bradykininogen

This term is used to describe a plasma protein which releases a kinin with properties like bradykinin during incubation with trypsin. "Kininogen" and "bradykinin precursor" are used as synonyms.

Unit

One unit of bradykininogen is the quantity which releases the equivalent of one µg synthetic bradykinin.

Specific activity

When one mg of protein releases kinin equivalent to x µg bradykinin, the preparation is said to have a specific activity of x µg bradykinin per mg of protein. One mg of this protein contains x µg. "potential bradykinin".

INTRODUCTION

The aim of the work now described has been the isolation from human plasma of the protein precursor of bradykinin. This is an essential preliminary to the characterisation of the protein and to the study of kinin-forming enzymes under controlled conditions.

The purification procedure has been designed to satisfy the following criteria as completely as possible.

1) The purified bradykinin precursor shall be free from kinin-forming enzymes and antagonists of these enzymes and also from kinin-destroying enzymes (kininases).

2) Conditions which carry a risk of denaturation shall be avoided.

3) The purified protein shall retain its biological activity under convenient storage conditions for long periods.

4) A high proportion of the kinin precursor present in fresh plasma shall be recovered.

5) The quantity of purified bradykininogen shall be adequate for the detailed study of activation by several enzyme systems and for the preliminary characterisation of the protein.

6) The final product shall be homogeneous as judged by

a) protein fractionation procedures with a high degree of resolution.

b) measurements of specific biological activity (μg bradykinin per mg protein).

Before describing the development of the method, the general background to the problem will be reviewed.

THE PLASMA KININS

Polypeptides with bradykinin-like properties released from plasma proteins have been termed plasma kinins. (Lewis, 1958). Recently five human plasma kinins have been distinguished by paper electrophoresis and chromatography. (Armstrong and Mills 1963). One of the group may be identical with the new kinin described by Elliot, Lewis and Smyth (1963).

1. CHEMISTRY

Bradykinin (Kallidin I)

H-Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg-OH.

Pure bradykinin (Bk) formed by the action of trypsin on acid-treated ox pseudoglobulin was isolated by Elliot, Lewis and Horton (1960 a.) after ethanol extraction, counter current distribution, chromatography on CM-cellulose and preparative paper electrophoresis. The same workers established the amino acid composition and sequence. (Elliot, Lewis and Horton 1960 b.)

Synthesis was achieved by Boissonas and his co-workers. The synthetic peptide was identical in biological activity with pure

ox bradykinin. (Boissonas, Guttmann and Jaquenoud 1960 (a) and (b); Boissonas, Guttmann, Jaquenoud, Konzett and Sturmer, 1960).

The molecular weight of the dihydrochloride of bradykinin is 1131 (Elliot, Horton and Lewis 1960).

Lysylbradykinin (Kallidin II)

H-Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg-OH.

When acid-treated human plasma was incubated with human urinary kallikrein (Pierce and Webster 1961) two plasma kinins were formed - kallidin I and II. Kallidin I was identical with bradykinin; kallidin II differed by the addition of lysine in the N-terminal position.

Bradykinin and lysylbradykinin were also formed by the action of bovine salivary kallikrein on plasma (Werle, Trautschold and Leysarth, 1961).

Lysylbradykinin was synthesised by Nicolaides, De Wald and McCarthy (1961).

Methionyllysylbradykinin

H-Meth.Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg-OH

Acid-treated ox pseudoglobulin at pH 7.5

released bradykinin, lysylbradykinin and a third kinin. Elliot, Lewis and Smyth (1963) isolated the latter and identified it as methionyllysylbradykinin.

Other plasma kinins

The euglobulin fraction of human plasma precipitated by 33% saturation with ammonium sulphate, formed kinins F and S spontaneously on incubation. They were separated and distinguished from bradykinin and lysylbradykinin by paper chromatography in butanol : acetic acid : water (4 : 1 : 5) and butanol : pyridine: water (1 : 1 : 1).

A kinin-forming enzyme from the euglobulin fraction acted on fresh plasma to form kinin E. (Armstrong and Mills, 1963).

One of the kinins E, F and S may be identical with methionyllysylbradykinin.

2. PHARMACOLOGICAL ACTIONS

The plasma kinins stimulate certain smooth muscles, produce vasodilatation, increase capillary permeability, cause chemotaxis of leucocytes and evoke pain from an exposed blister base.

The pharmacological actions of pure natural bradykinin were described by Elliot, Horton and Lewis (1960). The information summarised below was taken from these authors except where other references are given.

Actions on Smooth Muscle

The isolated guinea-pig ileum gave the characteristic slow contraction with 10^{-9} M bradykinin whereas the uterus of the stilboestrol treated rat responded to 10^{-10} M bradykinin. The dose response curve of the rat uterus was often very steep; a twofold increase in concentration could convert a threshold response into a maximum contraction.

Certain smooth muscles which showed rhythmic activity and a high resting tone were inhibited by bradykinin. The rabbit duodenum and the rat colon showed inhibition followed by stimulation. The rat duodenum showed only relaxation at a concentration of $8. 10^{-9}$ M.

The intravenous injection of 0.5 µg pure bradykinin in the anaesthetised guinea-pig caused bronchoconstriction comparable to that caused by 0.5 µg histamine. Bronchoconstriction was demonstrated by the method of Konzett

and Rossler (1940). A series of injections repeated at intervals of less than 10 minutes produced loss of sensitivity to bradykinin.

The bronchoconstrictor effects of bradykinin were antagonised by acetylsalicylic acid, phenylbutazone, amidopyrine, mefenamic acid and flufenamic acid at concentrations which did not affect the response to histamine or 5-hydroxytryptamine. (Collier and Shorley, 1960; Collier, 1963; Collier and Shorley, 1963).

Most normal men were unaffected by aerosols of 2% bradykinin in saline whereas 13 out of 15 asthmatic subjects developed dyspnoea and a 10 - 30% reduction of vital capacity with 0.5% bradykinin. (Herxheimer and Stresemann, 1961). Isolated rings of bronchial muscle were relatively insensitive to bradykinin.

Increased capillary permeability

The intradermal injection of bradykinin in the guinea pig after intravenous Pontamine sky blue produced intense local blueing. Elliot, Horton and Lewis showed that on a molar basis pure bradykinin was about 15 times as active as histamine. There were however qualitative

differences between the responses to the two agents.

Vasodilatation

100 ng bradykinin produced an increase in flow through the cat hind limb in vivo comparable to that produced by 10 - 20 ng acetyl choline.

Intravenous bradykinin lowered mean anterial pressure in the anaesthetised cat (400 ng/kg) and rat (150 ng/100 g).

Stimulation of pain fibres

Bradykinin (10^{-7} - 10^{-6} M) evoked transient pain from the base of a blister without the itching, flare and wheal which had been observed with crude plasma kinins. (Armstrong, Jepson, Keele and Stewart, 1957). When the concentration was increased 10-fold pain lasted for 2 - 3 minutes. After repeated doses of bradykinin higher concentrations were needed to evoke a comparable response. The desensitised area was unchanged in its sensitivity to histamine or 5-hydroxytryptamine.

The effects described above had all been observed previously with impure plasma kinins (Rocha e Silva 1955, Werle 1960, Roche e Silva

1960, Lahiri 1962, Lewis 1960).

Lysylbradykinin and methionyllysylbradykinin have the same actions as bradykinin; there are however quantitative differences between the three kinins (Table I). Full reports of the properties of kinins E, F and S have not yet appeared.

Table I

The relative potencies of the plasma kinins

(bradykinin = 1)

Assay	Lysylbradykinin (synthetic)	Methionyllysyl Bk (C) (purified)
rat uterus	1 (a)	¼
guinea-pig ileum	½ (d)	¼
rat duodenum	-	¼
intradermal blueing (guinea-pig)	-	1
bronchoconstriction (guinea-pig)	⅓ (b)	¼
vasodilatation (dog)	2 (a)	-
vasodilatation (cat)		¼
pain production (man)		¼

References (a) Webster and Pierce, 1963.

(b) Nicolaides, De Wald and Craft, 1963.

(c) Elliot, Lewis and Smyth, 1963.

(d) Habermann, 1963.

THE PLASMA KININ PRECURSOR

Rocha e Silva, Beraldo and Rosenfeld (1949) described the release of bradykinin from dog and ox plasma by trypsin and venoms from the genus Bothrops. The precursor of bradykinin was precipitated from plasma when the concentration of ammonium sulphate was increased from 30 to 45% saturation. This fraction, the classical pseudoglobulin, contained all the protein components of blood plasma but with relatively small quantities of albumin and γ-globulin. (Lewis 1960).

For several years plasma pseudoglobulin was used as the substrate for the study of kinin-forming enzymes. (Beraldo, 1950; Rocha e Silva, 1951; Hilton and Lewis, 1956; Hilton and Fox, 1956; Lewis, 1958). The pseudoglobulin from untreated serum or plasma contained both kinin-forming and destroying enzymes. Inactivation of these was achieved by heating the serum for 3 hours at 56°. This practice was introduced by Werle, Götze and Keppler (1937) as a method of inactivating serum kallikrein. During the heating a large part of the kinin precursor was lost however.

A further modification was introduced by Horton (1959). Dog plasma was brought to pH 2 by the addition of 1N HCl and incubated at 37° for 10 minutes. 1N $NaHCO_3$ was then added and pH 7.3 restored. Acid-treated dog plasma was free from kininase and virtually free from kallikrein. Horse plasma treated in this way contained active kallikrein however (Henriques, Picarelli and Ferraz de Oliveira, 1962).

Van Arman (1952) studied the bradykininogen content of bovine plasma fractions prepared by Cohn method 10. The bradykinin precursor was found in fraction IV and sub-fraction 4 contained twice as much potential bradykinin as sub-fraction 1. Bovine IV - 4 and human IV - 1 both contain kininase (Henriques et al, 1962, Erdös and Sloane 1962). Amongst the sub-fractions of bovine IV - 4, IV - 6 was most rich in bradykinogen (Van Arman 1955) but this fraction contained both kininase and kallikrein (Habermann, 1963).

During the paper electrophoresis of human plasma the plasma kinin precursor migrated as an α_2 globulin (Werle 1955).

Denatured bradykininogen remained vulnerable to trypsin; it was however resistant to the action of kallikrein (Diniz and Carvalho, 1963; Habermann, 1963). The two most recently introduced methods for the estimation of bradykininogen in whole plasma depend upon the quantitative release of bradykinin from denatured substrates by crystalline trypsin. The first of these was described by Diniz, Carvalho, Ryan and Rocha e Silva, 1961. Venous blood was taken in a siliconed syringe containing heparin. After centrifugation in polythene or siliconed glass, 0.2 ml plasma was added to 1.8 ml. 0.2% acetic acid and heated at 100° for 30 minutes. The denatured protein suspension was then neutralised with N NaOH and 0.5 ml. 0.2M. Tris buffer (pH 7.8) was added. After incubation with trypsin (200 µg/ml.) the bradykinin released was extracted with ethanol and assayed on an isolated smooth muscle preparation against standard bradykinin. 1 ml. human plasma contained potential kinin equivalent to 7.4 - 14.2 µg bradykinin.

A second method for the estimation of bradykininogen in heparin treated plasma was described (Brocklehurst and Lahiri 1962, Lahiri 1962). Protein precipitation in 80%

ethanol, at 0° was followed by heating at 80-90° for 20 minutes. The washed precipitate was incubated for 20 minutes with trypsin (500 µg/ml) in 0.1 M sodium phosphate buffer at pH 7.4. The trypsin was inactivated by heating and the kinin concentration determined by bioassay. Guinea pig plasma contained kinin precursor equivalent to 12.8 ± 1.9 µg bradykinin per ml.

In October 1961 when the work described in this thesis was started the information available about the bradykinin precursor was that summarised above. During the last two years four groups have published procedures for the purification of the bradykinin precursor, from horse plasma (Henriques, Picarelli, and Ferraz de Oliveira, 1962), from bovine plasma (Habermann and Rosenbusch 1962, Greenbaum and Hosoda 1963) and from human plasma (Webster and Pierce 1963). None of these methods fully satisfies the criteria set out in the introduction to this thesis.

Henriques et al treated oxalated horse plasma by the method of Horton (1959). The precursor was protected from the action of the activated endogenous kinin-forming enzyme by 20% saturation with ammonium sulphate.

The supernatant was fractionated by precipitation at 33 and 50% saturation. 1.7g of the precipitate at 50% saturation was dissolved in physiological saline, dialysed against 0.005 M sodium phosphate buffer, pH 7.0 and applied to a column of DEAE-cellulose (2x40 cm).

During gradient elution the bradykinin precursor left the column in 0.08 M sodium phosphate buffer 0.16 M with respect to NaCl. The active fractions were applied to a second DEAE column (1.1 x 38 cm) and gradient elution repeated. The final product contained 34% of the bradykinin precursor originally present in the acid-treated plasma and 0.2% of the original protein. 1mg of this protein gave the equivalent of 3.14 µg bradykinin with trypsin and 5.0 µg with the partially purified kinin-forming enzyme from the venom of Bothrops jararaca.

The bradykininogen is purified 170 fold by the method of Henriques et al. Acid-treatment carries a serious risk of denaturation however; the product may have different characteristics from the native protein. Protein estimations were made by a sensitive colorimetric method

(Lowry, Rosenbrough, Farr and Randall, 1951) which requires a standard protein for calibration. The authors do not state which protein was used so that direct comparison with the results of other workers is not possible. The yield of bradykininogen compares well with other methods. The product was said to be free from kinin forming enzyme and assumed to be free from kininase but no adequate data were given to support this. The heterogeneity of the purified protein was apparently not investigated.

Habermann and Rosenbusch (1962) applied Cohn method 6 to bovine plasma. They recovered about 80% of the total bradykininogen in fraction IV. Subfractionation gave most of this in fraction IV-6. This fraction was submitted to chromatography on calcium hydroxylapatite at pH 5.2 in acetate-phosphate buffer and eluted by continuous increase in ionic strength. The preparation released kinin with trypsin, Crotalus adamanteus venom and a kallikrein from pig pancreas. The kininogen was free from kinin forming enzyme but contained kininase. 1 mg of purified protein gave the equivalent of 10 - 20 µg bradykinin with trypsin.

Cohn fractionation appears to produce some denaturation; van Aman (1952) found that IV - 4 contained much insoluble protein. Habermann (1963) did not comment on the heterogeneity of the product nor did he state the method used for protein estimation.

Greenbaum and Hosoda (1963) developed a purification procedure for bovine bradykininogen which depended upon the persistent solubility of the protein at 100° in the presence of Ca^{++}. Bovine blood was defibrinated by whipping and then the serum was heated at 57.5° for 3 hours to inactivate peptidases. The serum was dialysed and ammonium sulphate added to 50% saturation. The serum globulin was dissolved in water, dialysed and heated in a boiling water bath. In the presence of calcium ion and Tris buffer at pH 7.7 a large amount of "inactive protein" precipitated as a coagulum. After centrifugation the supernatant was dialysed against 0.001 M sodium ethylenediaminetetra-acetate (EDTA) to remove Ca^{++}. Precipitation of bradykininogen took place when the pH was brought to 5.3. After repeated precipitation the bradykininogen was obtained as an acetone-dried powder.

The data given do not make it possible to calculate the overall recovery. The method gives less than 3% of the bradykinin precursor present in the crude globulin fraction however. 1 mg of the purified protein gave kinin equivalent to 0.8 - 1.0 µg bradykinin after incubation with trypsin. Free boundary electrophoresis at pH 8.6 and 7.5 gave one peak.

The method described by Greenbaum and Hosoda has the advantage that it can be applied to large volumes of blood. This is offset by the very low yield and furthermore the specific activity of the product is only one tenth of value given by Habermann et al. Free boundary electrophoresis gives poor resolution when compared with starch gel or immunoelectrophoresis and the low specific activity is evidence of heterogeneity; greater resolution might have been obtained by the use of pH values on different sides of the isoelectric point. The main disadvantage is the use of very extreme physical conditions which invite denaturation.

Webster and Pierce (1963) developed a method for the purification of the human kinin precursor from out-dated acid-citrate-dextrose plasma. Diluted plasma was adjusted to pH 6.0

and stirred with DEAE-cellulose at 18 - 20°C. This was filtered and the kinin precursor eluted in 0.3 M. potassium phosphate buffer pH 6.0. After dialysis and centrifugation the protein was added to a column of DEAE-cellulose (4.3 x 38 cm.) from which elution took place with a series of potassium phosphate buffers of increasing ionic strength; the kininogen was eluted by 0.12 M buffer. The active fractions were concentrated and applied to a column of hydroxylapatite (3.6 x 24 cm.) previously equilibrated with dilute potassium phosphate buffer, pH 6.8. Elution was again achieved by stepwise increase in potassium phosphate concentration. The main part of the kinin precursor was eluted at 0.039 M. 15% of the kininogen was recovered with 150-fold purification. However only 4% remained after dialysis, filtration and freeze drying.

The use of outdated plasma was unfortunate, since human plasma loses more than 75% of its kininogen over 4 days at 4° (Webster and Pierce, 1960). The loss of biological activity on final concentration may be due to leakage of kininogen through dialysis sacs or to the presence of kinin-forming enzyme.

In all species investigated the plasma kinin precursor is an α_2 globulin. The kininogen from bovine serum may have an isoelectric point of 5.3. There is no published information about molecular weight or the number of molecules of potential kinin present in one protein molecule.

Bradykinin, lysylbradykinin and methionyllysylbradykinin may be derived from the same protein molecule; the bradykinin sequence is common to all three kinins. The purest preparations of kininogen release bradykinin or lysylbradykinin with different kinin-forming enzymes (Habermann, 1963; Webster and Pierce, 1963). The substrate for kallikrein is exhausted by prior incubation with trypsin and vice versa (Habermann 1963).

The link between the polypeptides and the rest of the protein molecule may be via an ester linkage. Four possibilities were suggested by Elliot (1963).

1) An ester linkage between C-terminal Arginine and a carbohydrate part of the main protein.
2) An ester linkage between the - OH grouping of serine and a C-terminal arginine or lysine in the main protein.

3) An ester linkage between C-terminal Arginine and the -OH group of a serine component of the main protein.

4) A peptide linkage to the rest of the protein molecule.

Hamberg and Rocha e Silva (1957) showed that when snake venom was heated the bradykinin-releasing activity and the esterolytic activity on benzoyl-arginine methyl ester decreased slowly in parallel whereas proteolytic activity towards casein was lost much more rapidly. This suggests that kinin release depends on the hydrolysis of an ester bond.

ENZYMES WHICH RELEASE PLASMA KININS

The early work on the enzymes which release plasma kinins has been extensively reviewed (Frey, Kraut and Werle 1950, Werle 1955, 1960, Lahiri, 1962) and this section deals mainly with recent advances.

TRYPSIN

When trypsin acted on pseudoglobulin only bradykinin was detected although lysylbradykinin may have been formed as an intermediate. (Elliot, Lewis and Horton 1960 (a) and (b)).

Trypsin (20 µg/ml) released all the bradykinin from purified kininogen (10 mg/ml) in 120 minutes at 37° (0.10 M Tris buffer, pH 7.8, 0.2 M $CaCl_2$ was used for the incubation). An inverted exponential type of curve related kinin release to time (Greenbaum and Hosoda, 1963).

Diniz and Carvalho (1963) determined the optimum conditions for the release of bradykinin from denatured kininogen. The potential kinin

in 0.2 ml. plasma was completely released by trypsin (200 µg/ml) in 30 minutes at pH 7.8 and 37°. The enzyme was more active at this pH than at 7.4 or 8.2.

KALLIKREIN

This term was used by Frey and Kraut (1928) to identify an agent in urine which caused hypotension on intravenous injection. Urinary kallikrein was later shown to act by the release of kinin. Several different enzymes with this action have now been demonstrated in tissue fluids and extracts of exocrine glands.

Highly purified kallikreins have been prepared from the following sources; pig pancreas (Habermann, 1963; Moriya, Pierce and Webster, 1963; Werle and Trautschold, 1963); pig salivary gland, urine and serum (Werle and Trautschold, 1963); horse urine (Prado, Prado, Brandi and Katchburian, 1963); bovine pancreas (Webster, Clark and Anderson 1956); human plasma, pancreas and urine (Webster and Pierce, 1963; Moriya, Pierce and Webster, 1963).

Human kallikreins obtained from different sources differed in physical properties, immunological properties, enzyme activity and sensitivity to inhibitors.

Purified kallikreins from human urine and pancreas had molecular weights of 40,500 and 31,200 respectively; the urinary kallikrein migrated more rapidly than the pancreatic enzyme during electrophoresis in Cyanogum gel (Moriya et al, 1963).

A rabbit antiserum to crude human urinary kallikrein inhibited the vasodilatation produced in the dog by pure human urinary kallikrein; the response to pure human pancreatic kallikrein was also inhibited. In agar gel the antiserum gave single, precipitin lines with the pure kallikreins from urine and pancreas. These two lines became continuous. No comparable line was given by human serum and there was no cross reaction with kallikreins from dog urine or pig pancreas (Webster, Emmant, Turner, Moriya and Pierce, 1963).

Kallikrein from human urine released only lysylbradykinin from purified human kininogen

whereas human plasma kallikrein released only bradykinin. Neither enzyme produced bradykinin from synthetic lysylbradykinin (Webster and Pierce, 1963). Human and pig pancreatic kallikrein and pig salivary gland kallikrein resembled human urinary kallikrein (Webster and Pierce, 1963; Werle, Trautschold and Leysarth, 1961).

Crude kallikrein preparations were proteolytic but highly purified kallikreins from pig pancreas, pig salivary gland and human urine had negligable proteolytic activity on casein or haemoglobin. These enzymes had esterolytic activity however which was strictly limited to certain esters of benzoyl-L-arginine and p-tosylarginine. The hydrolysis of benzoylarginine ethyl ester (BAEE) was used for the measurement of kallikrein activity (Werle and Trautschold, 1963).

Purified pig serum kallikrein retained both proteolytic and fibrinolytic activity. Werle and Trautschold suggested that the preparation was contaminated with plasmin. The kallikrein of serum or plasma may however be able to hydrolyse a range of both ester and peptide links.

The selective inhibition of kallikreins from different sources is described in the section on kallikrein inactivators.

When acid-treated ox pseudoglobulin was incubated at pH 7.5 in the absence of exogenous enzyme, bradykinin and lysylbradykinin (Habermann and Okon, 1961; Elliot, 1963) and methionyllysylbradykinin (Elliot et al, 1963) were formed. One or more kinin-forming enzymes had been activated.

Human plasma euglobulin (precipitated at 33% ammonium sulphate saturation) formed plasma kinins F and S spontaneously. A kinin-forming enzyme purified from this fraction released kinin E from fresh plasma (Armstrong and Mills, 1963). This enzyme was inhibited by hexadimethrine bromide but not by ϵ-amino-n-caproic acid (Armstrong and Mills, 1962); it may differ from both plasma kallikrein and plasmin.

THE ACTIVATION OF KALLIKREINOGEN

The kallikreins of plasma and pancreas occur as inactive precursors. Activation of plasma

kallikreinogen has been described after the following treatments; acidification of serum with HCl to pH 2.0 at 0° (Kraut, Frey and Werle, 1933); incubation of horse plasma at pH 2.0 and 37° for 15 minutes (Henriques et al, 1962); incubation with trypsin or papain (Werle 1955); exposure of serum to 20% acetone for 4 hours (Webster and Pierce, 1960); treatment with acetophenone or benzophenone (Frey, Kraut and Werle, 1950); treatment with chloroform (Lewis 1958).

Pancreatic kallikreinogen was extracted from pancreatic tissue in which autolysis had been suppressed. This proenzyme was activated by trypsin. Similar proenzymes were obtained from brain and intestine (Werle, 1960).

The active sites of these kinin-forming proenzymes may be masked by additional groups which dissociate at low pH and in the presence of certain organic solvents. In other situations the masking groups may be removed by hydrolytic enzymes such as plasmin or trypsin. It is possible that the final step in the activation of plasma kallikreinogen is always enzymic, although a variety of physical conditions initiate the process.

The kallikreinogens of plasma and pancreas could be activated normally after incubation with DFP and removal of the excess. The corresponding kallikreins lost their hypotensive, kinin-forming and esterolytic activity however in the presence of DFP. These inactivated kallikreins no longer bound the naturally occurring inactivators described in the next section. It was suggested that a serine unit was present in the active site of the enzyme and that this was essential for kinin-forming activity and combination with the masking group or inactivator (Werle and Trautschold, 1963).

KALLIKREIN INACTIVATORS

Werle and Trautschold (1963) described two kallikrein inactivators in serum which could be distinguished by their pH optima and solubilities in perchloric acid. Other inactivators occurred in extracts of parotid salivary gland, liver, lymph gland, posterior pituitary, potatoes and egg white. Some non-specific antagonists of proteolytic enzymes; others were polypeptides with a high specificity for the kallikreins (Werle, 1955).

Many kallikreins were inhibited by incubation with DFP (Habermann, 1960; Werle and Trautschold, 1963). Webster and Pierce (1963) described the pattern of inhibition of purified human kallikreins. Soya bean trypsin inhibitor (SBTI) was effective only against plasma kallikrein and egg white against urinary kallikrein; kallikreins from plasma, pancreas and urine were all inhibited by pancreatic trypsin inhibitor (PTI) but unaffected by ovomucoid trypsin inhibitor (OTI).

Several analgesic-antipyretic drugs inhibit kinin-formation in vitro. Phenylbutazone, 2:6 dihydroxybenzoic acid, sodium α(4-sec-butylphenoxy) propionate, salicylate and acetyl-salicylate inhibited the kallikreins of guinea-pig serum and human saliva at concentrations which did not interfere with the bioassay of kinins (Northover and Subramanian, 1961).

PLASMIN

Two plasminogens, designated A and B, have been detected in human plasma by immuno-electrophoresis in fibrin-agar plates (Rybak and Petakova, 1963). These plasminogens have no proteolytic activity; activation is essential

before the proteolytic properties of plasmin appear.

Plasmin hydrolyses fibrin, fibrinogen, casein, haemoglobin, tosylarginine methyl ester (TAME) and lysine ethyl ester (Sherry, Fletcher and Alkjaersig, 1959). The enzyme is inhibited by DFP (Mounter and Shipley, 1958), an antiplasmin from Cohn IV-1 (Siegel, Barclay and Cliffton, 1956), several trypsin inhibitors and ε-aminocaproic acid (Alkjaersig, Fletcher and Sherry, 1959).

A large number of agents activate plasminogen; plasmin itself (Mounter and Shipley, 1958); trypsin, urokinase, streptokinase (Alkjaersig, Fletcher and Sherry, 1958); enzymes in blood, skin and lung; chloroform; silica surfaces; ammonium sulphate. This subject has been reviewed by Sherry et al, (1959). Many of these agents also activate plasma kallikreinogen.

The release of plasma kinin by plasmin was first described by Beraldo (1950). Human plasmin released kinin from dog pseudoglobulin (Lewis and Work, 1957; Lewis, 1958) and preactive human plasma (Eisen, 1961). This was prevented by an antiplasmin and by SBTI

(Lewis, 1958; Schachter, 1960).

Intravenous plasmin lowered arterial blood pressure in several species including man (Back, Guth, and Munson, 1963). Tachyphylaxis developed; Back et al showed that this was not due to depletion of kininogen.

Eisen (1963a) compared the relative effects of different activating treatments on the fibrinolytic and kinin-forming systems of human plasma. Silica surfaces, acidification and acetone precipitation produced rapid release of kinin with low rates of fibrinolysis. Ammonium sulphate precipitation and streptokinase favoured fibrinolysis rather than kinin formation. ϵ-aminocaproic acid did not prevent the development of kinin-forming activity in plasma exposed to glass (Armstrong and Stewart, 1960), low pH, acetone or chloroform (Eisen, 1963). Eisen concluded that the kinin-forming system of plasma was probably independent of the fibrinolytic system.

Using kallikrein-free human plasmin Vogt (1963) observed kinin release from unheated dog plasma globulin but not from heated globulin (56° for 2 hours) or purified bovine kininogen.

He concluded that plasmin released kinin indirectly by the activation of plasma kallikreinogen.

SURFACE ACTIVATION OF PLASMA KININS

When plasma was brought into contact with glass, kinin was released (Armstrong, Jepson, Keele and Stewart, 1957). The complex sequence of reactions responsible has been studied in detail (Keele 1960, Eisen 1963(a), Margolis 1963).

On exposure of plasma to negatively charged surfaces (glass, kaolin, alumina and others) Hageman Factor was adsorbed and the physical state of this basic protein altered so that it became capable of activating component A. Activated component A released kinin from its precursor in the presence of an essential cofactor (Component B). (Keele 1960, Margolis 1963).

Activated Hageman Factor may be responsible for the initiation of blood clotting and fibrinolysis.

The nature of components A and B is not well established. The actions of component A were

prevented by heating at 56° for 2 hours and by SBTI, and it may be identical with plasma kallikrein (Eisen 1963a). Plasma could be depleted of component B under the action of activated component A (Keele 1960).

ACTIVATION OF PLASMA KININS BY DILUTION

The release of kinin on dilution of plasma or serum was described by Schachter (1956). The optimum dilution, the quantity of bradykinin-like activity detected and the rate of inactivation varied with the species.

Dilution appeared to activate endogenous kinin forming enzymes and to reduce the effectiveness of the plasma kallikrein inactivators. Eisen (1961) reported that both glass activation and dilution activation were due to the same enzyme.

SNAKE VENOMS

The kinin-forming enzyme from Bothrops jararaca was purified by selective denaturation and ammonium sulphate fractionation. Proteolytic activity decreased during purification (Henriques, Picarelli and Oliveira 1962).

Venoms from Agkistrodon contortrix and piscivorus (Webster and Pierce, 1963) and Crotalus adamanteus (Habermann 1963) released bradykinin from purified preparations of kininogen.

ENZYMES WHICH INACTIVATE THE PLASMA KININS

The plasma kinins are hydrolysed by several proteolytic enzymes; biological activity is usually reduced or abolished.

TRYPSIN

Trypsin hydrolysed peptide or ester links formed from the carboxyl groups of L-arginine or L-lysine. When other amino acids replaced arginine or lysine in synthetic substrates the peptide became resistant to trypsin. (Bergmann and Fruton, 1941; Dixon and Webb, 1958a).

Trypsin acted on methionyllysylbradykinin and lysylbradykinin to give bradykinin (Elliot, Lewis and Smyth, 1963; Erdös, Rendrew, Sloane and Wohler, 1963; Nicolaides, DeWald and Craft, 1963). Bradykinin itself was stable in the presence of trypsin (Rocha e Silva, 1955) unless the concentration was high (Diniz and Carvalho, 1963). Inactivation by high concentrations of trypsin was probably due to contamination with carboxypeptidase or

chymotrypsin (Desneuelle and Rovery, 1961). The atypical imino acid proline probably protected the N-terminal exopeptide bond from hydrolysis by trypsin.

CHYMOTRYPSIN

Chymotrypsin hydrolysed peptide or ester linkages formed from the carboxyl groups of phenylalanine or tyrosine (Bergmann and Fruton, 1941); in its presence bradykinin lost its biological activity (Rocha e Silva, 1955) and was split into three parts. These were identified as arginylprolylprolylglycylphenylalanine, serylprolylphenylalanine and arginine (Elliot, Lewis and Horton, 1960 b).

CARBOXYPEPTIDASE B

The basic carboxypeptidase inactivated bradykinin and lysylbradykinin (Erdös, Renfrew, Sloane and Wohler, 1963). The enzyme was specific for an exopeptide bond involving a C terminal arginine or lysine unit (Folk and Gladner, 1958 a and b); it may be responsible for the kininolytic activity of commercial preparations of pancreatic kallikrein. The loss of biological activity on exposure to pure

carboxypeptidase B confirms that the activity was due to a bradykinin-like peptide.

Low concentrations of chymotrypsin and carboxypeptidase B had identical effects on the bradykinin molecule. Chymotrypsin however inactivated most biologically active peptides, (Gaddum, 1955).

PLASMA KININASE

Plasma fractions which contained bradykininogen commonly contained kininase (Werle 1955; Lewis 1960; Henriques, Picarelli and Ferraz de Oliveira 1962; Habermann 1963).

Horton (1959) demonstrated that the kininase of dog plasma was inactivated by incubation at pH2 and 37° for 20 minutes.

Kininase activity was found in the Cohn IV-1 fraction of human plasma (Erdős, and Sloane, 1962; Erdős, Renfrew, Sloane and Wohler, 1963). The fraction behaved as a carboxypeptidase splitting off the C-terminal arginine from synthetic bradykinin or lysylbradykinin. The kininase was distinguished from pancreatic carboxypeptidase B however by the different susceptibilities of the

two enzymes to inhibitors (Table II). Erdös et al called the enzyme "carboxypeptidase-N".

When the carboxypeptidase of human plasma had been inhibited by manganese ions, additional proteolytic activity was demonstrated; the N-terminal peptide bond of lysylbradykinin was split by an aminopeptidase (Erdös et al, 1963).

RED CELL ENZYMES

During incubation with lysed red cells, synthetic bradykinin was split into its amino acid components and the arginine units were converted to ornithine (Erdös et al, 1963).

TISSUE KININASE

Bradykininolytic enzymes have been reported in preparations from kidney and liver (Hamberg and Rocha e Silva 1954; Werle 1955).

Brocklehurst and Lahiri (1962) described a kininase released during perfusion of guinea pig lung with Tyrode solution. The rate of release was uninfluenced by an antigen-antibody reaction. Activity was lost in 30 minutes at 56° But SBTI had no effect. The optimum pH for the inactivation of synthetic bradykinin lay between 7.0 and 7.5.

Table II

Selective inhibitors of kinin-destroying enzymes (Erdös et al, 1963)

Enzyme	Chymotrypsin	Carboxypeptidase-B	Carboxypeptidase N
Source	pancreas	pig pancreas	plasma IV-1
Substrate		hippuryl-L-arginine	synthetic bradykinin
EDTA	0	0	+
DFP	+		0
SBTI	+		0
OTI	+		0
Cysteine			+
ϵ-amino-n-caproic acid		+	+
6M urea		0	+
$MnCl_2$			+
$CoCl_2$			-

(+ = inhibition, 0 = no effect, - = potentiation).

II EXPERIMENTAL

THE PURIFICATION OF BRADYKININOGEN BY PRECIPITATION METHODS

INTRODUCTION

Fractional precipitation methods have been used in the purification of kininogens from horse and ox blood (Henriques et al, 1962; Greenbaum and Hosoda, 1963). These procedures have the important advantage that they can be applied to large volumes of plasma.

Three methods have been investigated.

1. fractional extraction with ammonium sulphate solutions,
2. fractional precipitation with potassium phosphate buffers, and
3. Cohn fractionation.

Each has failed to satisfy many of the criteria listed in the introduction to this thesis.

THE FACTORS WHICH DETERMINE THE SOLUBILITY OF A PROTEIN.

The effect of salt concentration on the solubility of a single protein was investigated by Cohn; he derived the following expression,

$\log.S = \beta - K_s . I/2$ (Cohn, 1925)

where S is the protein solubility in grams per Kg. water, I/2 is the ionic strength in moles per Kg. water and K_s and β are constants. Dixon and Webb (1961) derived the modified expression,

$\log.s = \beta' - K'_s . \lambda/2$

where s is expressed in g/litre and λ in moles/litre of solution.

The intercept constant β' is the theoretical log. solubility in the absence of salt; when pH, temperature and dielectric constant are controlled, it is characteristic of the protein.

K'_s is the "salting-out" constant - the slope of the line relating log. solubility to ionic strength. It is characteristic of the protein-salt pair, and independent of pH and temperature.

<u>The importance of protein concentration</u>

Salt may be added to a protein solution without precipitation, until the protein solubility no longer exceeds the protein concentration. The solution is then saturated with protein and the lower precipitation limit has been reached. This point is constant

for a given protein only when its concentration is constant.

Butler and Montgomery (1932) diluted plasma before precipitation to minimise protein associations and co-precipitation, the evidence for association in solutions of high ionic strength is unconvincing however (Dixon and Webb, 1961). High dilutions not only reduce the amount of protein which can be handled, but also necessitate salt concentrations which approach saturation and interfere with the sedimentation of precipitates.

The choice of salt

When salt is added to the saturated protein solution the solubility falls and protein precipitates. The increase in salt concentration for nearly complete precipitation is inversely related to the slope of the log. solubility curve; a high K_s' is required for narrow precipitation limits and high resolution.

In experiments with carboxyhaemoglobin the K_s' values of common salts decreased in the order, potassium phosphate, sodium sulphate, ammonium sulphate, sodium citrate, magnesium sulphate,

sodium chloride (Dixon and Webb, 1961). Sodium sulphate has a low solubility relative to ammonium sulphate and potassium phosphate which were chosen for the experiments on human bradykininogen.

The relative positions of the precipitation limits of two proteins are altered by dilution; they may be separated or approximated. The effect of dilution can only be predicted if K'_s is known for each protein.

The effect of pH

A protein is least soluble at its isoelectric point. In other parts of the pH range a change of one unit may increase solubility tenfold. The relative solubility of two proteins with different isoelectric points may be altered by a factor of several hundred.

The effect of temperature

β' varies inversely with temperature; between 0° and 25° the solubility of carboxyhaemoglobin falls by a factor of ten. Ovalbumin has minimum solubility at 25° but this is exceptional.

When designing a method other factors influence the choice of temperature. The solubility of the salt and the stability of the protein must be considered.

Dielectric constant

Ethanol and acetone are extensively used in the fractionation of plasma proteins (Askonas, 1951). In their presence the dielectric constant of the solution is reduced and protein precipitates. These effects are opposed by glycine.

Heavy metal ions

Metallic cations reduce protein solubility; certain proteins form soluble complexes however.

Fractionation with ammonium sulphate

Ammonium sulphate is highly soluble and has a moderate K'_s value. The risk of denaturation is small and the salt makes certain dissolved proteins more stable (Dixon and Webb, 1958).

When horse serum proteins were fractionated by ammonium sulphate, the ionic strength was increased slowly and smoothly by diffusion across collodion membranes. The fractions were classified by their behaviour on electrophoresis.

Fractionation of horse serum

(Cohn, McMeikin, Oncley, Newell and Hughes, 1940)

ammonium sulphate (moles/l)	fraction
1.39	γ globulin
1.64	α, β and γ globulins
2.05	α, β globulins and mucoproteins
2.57	albumin
2.80	albumin and glycoproteins

(pH 6, room temperature)

Zahn and Stahl (1955) saturated human plasma with solid ammonium sulphate. The protein precipitate was mixed with kieselgur, supported in a Büchner funnel and extracted by an ammonium sulphate solution of continuously decreasing concentration. Several partially purified fractions were resolved. The effectiveness of the method was confirmed by the separation of bovine serum albumin from human haemoglobin (Zittle and Della-Monica, 1955).

Ammonium sulphate has no buffering capacity. Different batches of commercial preparations (Analar grade) give different pH values in solution. The glass electrode does not give reliable readings in solutions above 0.3M (Dixon and Webb, 1961).

Fractionation with potassium phosphate

Concentrated phosphate buffers are effective precipitating agents; the value of K'_s is high and narrow precipitation limits can be obtained. Cohn (1927) obtained data which made it possible to prepare a series of potassium phosphate buffers of constant pH but graded ionic strength. Green and Hughes (1955) published such data in tabular form.

Butler and Montgomery (1932) investigated the solubility of plasma proteins in potassium phosphate buffers at pH 6.5. Human plasma at a dilution of 1:16 and 25° gave a complex solubility curve with breaks in continuity at 1.1 and 2.4M. These points corresponded with nearly complete precipitation of fibrinogen and globulin respectively.

Fractional precipitation with potassium phosphate buffers permits better control of conditions than is possible with ammonium sulphate.

Fractionation with ethanol

During the early stages of development of the Cohn procedures ammonium sulphate was replaced by ethanol. Human plasma gave four

fractions rich in fibrinogen, γ globulin, αβ globulin and albumin. Denaturation was minimised at 0° to -5° and the ethanol was removed during freeze-drying (Cohn, Leutscher, Oncley, Armstrong and Davis, 1940). In 1946 when "Method 6" was published six main fractions and several subfractions had been obtained (Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946).

"Method 10" was published in 1950; protein fractions were precipitated close to their isoelectric points under accurately controlled conditions of temperature, pH, ionic strength, dielectric constant and cation concentration. Albumin (V), γ-globulin (II) and fibrinogen (I) were obtained in high purity. The more complex fractions (III) and (IV) were resolved into multiple subfractions (Cohn, Gurd, Surgenor, Barnes, Brown, Derouaux, Gillespie, Kahnt, Lever, Liu, Mittleman, Mouton, Schmid and Uroma, 1950).

The occurrence of bovine bradykininogen in IV-4 (method 6) and IV-6 (method 10) (van Arman, 1952, 1955) has been described earlier.

EXPERIMENTAL

FRACTIONAL EXTRACTION WITH AMMONIUM SULPHATE SOLUTIONS.

A simplified version of the fractional extraction method of Zahn and Stahl (1955) was applied in the purification of bradykininogen. It was hoped that kininogen would be extracted after the removal of kallikrein or under conditions in which kallikrein remained insoluble. Loss of kininogen due to the action of kallikrein would then be prevented.

The plasma globulins were precipitated in 3M ammonium sulphate at 4°. Protein fractions were extracted from the precipitate by a series of increasingly dilute solutions of ammonium sulphate.

In small scale experiments the precipitate was packed in the refrigerated centrifuge, drained and re-suspended in the extracting solution. In preparative experiments the extracting solution was passed through a layer of precipitate mixed with a filter-aid.

Small scale experiment

1 ml aliquots of plasma were added to 3 ml volumes of 4M ammonium sulphate. After 12 hours at 4° the precipitates were packed and each was suspended in 5 ml of an ammonium sulphate solution of known concentration. The precipitate which remained after extraction for one hour at 4° was suspended in ethanol and the bradykininogen content determined by the method of Brocklehurst and Lahiri (1962). The results are tabulated below.

ammonium sulphate (moles/l)	bradykininogen remaining in precipitate (units)
2.2	7.8
1.8	6.5
1.4	2.3
1.0	0.4

Most of the bradykinin precursor failed to dissolve in 1.8M ammonium sulphate but little remained insoluble at 1.0M.

Preparative experiment

20 ml plasma were added to 60 ml 4M ammonium sulphate at 4°. After equilibration as before the precipitate was mixed with 8 - 10 g

G-50 Sephadex and filtered under gravity in a polythene Büchner funnel. A second filter paper was applied after a firm layer had formed and the precipitate was washed with 2.0M ammonium sulphate. Six fractions were extracted in 100 ml volumes, the concentration being reduced in 0.2M steps. The bradykininogen content of each fraction was estimated as before.

ammonium sulphate (moles/l)	bradykininogen in extract (units)
1.8	4
1.6	16
1.4	10
1.2	5
1.0	<1
0.8	<1

After 5 days in phosphate buffer at pH 7.6 and 4° only the 1.2 M fraction contained more than one unit of bradykininogen. The method appeared to offer little prospect of kallikrein-free, kininogen-rich fractions. Less than 20% of the bradykininogen contained in 20 ml of human plasma (Diniz et al, 1961) was recovered.

Repeated attempts were made to increase the yield of kininogen. The pH of the ammonium

sulphate solutions was adjusted to 6.0 and 4.0 in different experiments and the protein fractions were precipitated immediately after extraction. There was little improvement however and the method was not developed further.

FRACTIONAL PRECIPITATION WITH POTASSIUM PHOSPHATE BUFFERS

Potassium phosphate buffers were used in an attempt to develop a fractionation method with higher resolution and narrower precipitation limits than those provided by ammonium sulphate.

Precipitations were carried out at pH 5.8 and 20°. pH 5.8 was chosen because this was the pH for precipitation of IV-4 (Cohn et al, 1946) and the presumed isoelectric point of the bradykinin precursor. The poor solubility of potassium phosphate at low temperatures made it necessary to work at 20°. Plasma was diluted 1:11 to minimise co-precipitation.

Phosphate buffers of graded ionic strength but constant pH were prepared according to the data of Green and Hughes (1955) and Cohn (1927), (Appendix 8). The molarities given below take account of the dilution of buffer by plasma.

Small scale experiment

1 ml aliquots of plasma were added to 10 ml volumes of potassium phosphate buffer; the mixtures were shaken for 12 hours at 20°. The bradykininogen contents of the precipitates were determined after filtration.

potassium phosphate (moles/l)	bradykininogen in precipitate (units)
2.6	15
2.4	15
2.2	15
2.0	11
1.8	5
1.6	<1
1.4	<1

Preparative experiment

The first precipitation was carried out at 1.6 M, 50 ml of plasma having been added to 500 ml 1.76 M buffer. 3 hours were allowed for equilibration, then the precipitate was removed in a filter.

The filtrate was transferred to a dialysis sac which was immersed in three volumes of 2.4 M buffer. 24 hours were allowed for complete

precipitation at the final molarity of 2.2. Donnan effects and volume changes on mixing were neglected.

The precipitate at 2.2 M contained 20 - 40 units bradykininogen; this represents about 4% of the theoretical yield predicted by the small scale experiment. This loss was attributed to the action of plasma kallikrein which apparently remained active in 1.6 M phosphate buffer at pH 5.8.

COHN FRACTION IV-4

The specific activity of human IV-4 (Method 6) prepared from outdated acid-citrate-dextrose blood was determined. Trypsin released kinin equivalent to 0.06 µg bradykinin from 1 mg of the freeze-dried protein. The specific activity of total plasma protein was 0.10 - 0.20 µg bradykinin/mg protein* (Diniz et al, 1961). Large losses of kininogen had occurred during storage of the blood or during the Cohn procedure.

IV-4 was used in a preliminary experiment with ion-exchange chromatography but it was

*A plasma protein concentration of 75 mg/ml is assumed.

not considered a suitable starting point for further purification. IV-4 from siliconed fresh plasma was not available.

THE PURIFICATION OF BRADYKININOGEN BY ION-EXCHANGE CHROMATOGRAPHY

INTRODUCTION

Precipitation procedures failed to resolve kininogen and kallikrein, and serious loss of kininogen thus occurred. It was therefore decided to fractionate plasma by column chromatography without preliminary purification.

THE COLUMN CHROMATOGRAPHY OF PROTEINS

Ion-exchange and adsorption chromatography provide preparative methods with high resolution.

Ion-exchange on synthetic resins was introduced as a means of separating fission products and rare earth mixtures. The ions penetrated the resin particles gaining access to numerous exchange sites. (Spedding, Fulmer, Butler and Powell, 1950).

The weak cation-exchange resin, Amberlite IRC-50, was used to separate stable proteins of low molecular weight and high isoelectric point (Zittle, 1953). Protein molecules did not penetrate however and fine division of the

particles was necessary to expose enough binding sites. This produced high resistance to flow. Some proteins were bound so firmly that the molecules were distorted and the conditions needed for elution caused denaturation.

Calcium hydroxylapatite ($Ca_5(PO_4)_3OH$) was used in the fractionation of albumins, globulins and other proteins (Tiselius, Hjerten, and Leven 1956). Columns have been used to purify plasma kinin precursors (Habermann and Rosenbusch, 1962; Webster and Pierce, 1963). The exact nature of the binding sites is unknown.

The introduction of cellulose adsorbents was a major advance (Peterson and Sober, 1956). They provided hydrophilic supports with large surfaces for protein binding. Diethylaminoethyl (DEAE-) cellulose and carboxymethyl (CM-) cellulose were derived by substitution with 2-chlorotriethylamine hydrochloride and chloroacetic acid respectively. DEAE-cellulose had an anion capacity of 0.18 to 1.98 mE/g and a pK' of about 9.5 in 0.5 M NaCl. CM-cellulose had a cation capacity of 0.67 to 1.57 mE/g and a pK' between 3.8 and 4.3 (Peterson and Sober, 1956).

DEAE- and CM- derivatives of the cross-linked dextran gels, G-25 and G-50 Sephadex,

became available during 1960/61. "DEAE-Sephadex A-50" is penetrated by proteins comparable in size to pepsin (35,000); the capacity for anion is 3 - 4 mE/g and the pK' in 1 M KCl about 8. CM-Sephadex C-50 has a cation capacity of 4 - 5 mE/g and a pK' about 4. Under appropriate conditions the gel from 1 g of dry powder will bind 1 - 2 g protein. The Sephadex ion-exchange derivatives are more homogeneous than the corresponding cellulose derivatives and have convenient handling qualities.

Factors influencing adsorption

Binding occurs at pH values between the isoelectric point of the protein and the pK' of the ionising groups. The DEAE- derivatives are effective below pH 8 and the CM- derivatives above pH 4. The affinity for the ionised groups is determined by the number and distribution of the charges on the protein molecule. A large protein with a high charge density is most firmly bound; high local concentrations of charge favour binding.

The presence of electrolytes influences protein adsorption; when the ionic strength of the medium is moderate or high, the protein

binding capacity of the adsorbent is reduced or abolished. The preparation of plasma protein for adsorption commonly requires reduction of ionic strength and pH adjustment. When the initial adsorption is to be selective, the adjustment must be exact; this may be achieved by dilution, dialysis or gel-filtration. Dilution is often satisfactory when the plasma electrolyte composition is known. Dialysis is slow. Dextran-gel filtration gives rapid and complete equilibration with buffer of chosen pH and ionic strength, and will usually remove some unwanted constituents concomitantly.

Selective elution

Protein is eluted when the association with the binding sites is weakened by increased ionic strength or pH change. Both of these variables are used to obtain selective elution. This should be achieved without recourse to extreme conditions.

The conditions for high resolution are well established (Peterson and Sober, 1962). The protein load should be small by comparison with the total capacity of the column. The sample

should be applied in small volume or under conditions which allow tight adsorption to a long column of uniformly packed fine particles. The ionic strength or pH should be altered in a continuous gradient and not in discrete steps. This takes full advantage of adsorption equilibrium, the state in which partially adsorbed protein moves slowly down the column.

Stepwise elution with a graded series of buffers does not give high resolution but offers advantages in other ways. Large loads may be fractionated on short, wide columns with coarse particles and high flow rates. Rapid separation of components with different adsorption characteristics occurs, e.g. an enzyme may be separated from its substrate.

The fractionation of plasma proteins

The following examples of plasma protein fractionation by column chromatography served as a guide to the choice of conditions.

An inclusive fractionation scheme using gradient elution from DEAE-cellulose has been described (Sober, Gutter, Wyckoff and Peterson, 1956). Selective elution was produced by continuous increase in ionic strength and

decrease in pH; the proteins left the column in decreasing order of isoelectric point. The fractions were partly characterised by paper electrophoresis; certain proteins were identified more exactly. Proteins migrating as α_2 globulins appeared in fractions 12 - 16 (gradient to 0.02 M NaCl, 0.05 M NaH_2PO_4) and again in fractions 21 - 29 (gradient to 0.10 M NaCl, 0.05 M NaH_2PO_4). The fast α_2 component, caeruloplasmin, appeared in fraction 28. Albumin appeared throughout the latter half of the chromatogram (15 - 30).

Human serum was fractionated on DEAE-Sephadex A-25 equilibrated with 0.02 M phosphate buffer pH 6.6. Elution was achieved by stepwise increase in NaCl concentration. (Lundbland, 1962).

Hogmann and Killander (1962) separated γ-globulins on DEAE-Sephadex A-50 in phosphate buffer at pH 8.0. The components of a plasma protein fraction prepared by gel-filtration were resolved by gradient elution from DEAE- Sephadex at pH 8.0 (Gelotte, Flodin and Killander, 1962).

Flodin (1961) described the behaviour of human serum proteins on DEAE-Sephadex A-50 in 0.05 M phosphate buffer, pH 6.6. γ-globulin and a β-component were not adsorbed; albumin, caeruloplasmin and the 19 S antibodies were eluted by stepwise increase in NaCl concentration. The non-adsorbed fraction was equilibrated with 0.04 M acetate buffer, pH 5.8 and applied to CM-Sephadex. Three γ-globulins and transferrin were eluted in separate peaks by increasing concentrations of salt. Such a combination of anion and cation-exchange steps has been recommended by Peterson and Sober (1962).

EXPERIMENTAL

THE CHOICE OF ION-EXCHANGE MATERIAL

DEAE-Sephadex became available shortly before this work was started and it appeared to have advantages over DEAE-cellulose. Preliminary experiments with Cohn fraction IV-4 and diluted whole plasma suggested that it could be useful in the purification of bradykininogen.

75 mg IV-4 (4.5 units bradykininogen) were applied to a column of DEAE-Sephadex A-50 (1.3 x 20 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5, 0.02 M-NaCl). The protein was eluted in 1 litre by continuous increase in sodium chloride concentration. Bradykininogen (>3 units) was eluted between sodium concentration limits of 0.16 and 0.28 E/l.

THE PREPARATION OF PLASMA FOR CHROMATOGRAPHY

It was found necessary to reduce the ionic strength of plasma before chromatography. Three methods of doing this were tried.

1. Dilution

3 ml plasma were diluted to 20 ml with cold 0.02 M phosphate buffer, pH 7.6 and applied to a column of DEAE-Sephadex (2.4 x 10 cm) at 4°. The salt concentration of the eluant was increased in steps and five 250 ml fractions were collected. The bradykininogen content of each is shown below.

Fractionation of 3 ml plasma on DEAE-Sephadex A-50

0.02 M sodium phosphate, pH 7.6, 4°.

Sodium chloride (moles/l)	Bradykininogen in eluate (units)
0.10	0.5
0.14	0.6
0.18	4.0
0.22	1.3
0.30	<1.1

The bradykinin precursor was adsorbed at concentrations below 0.14 M but eluted when the concentration was increased to 0.18 M.

20 ml plasma was added to a stirred suspension of DEAE-Sephadex (12 g) in 180 ml 0.02 M phosphate buffer (0.08 M-NaCl) at 4°. The suspension was filtered, packed into a column and eluted as above. Bradykininogen (18 units) was eluted between 0.10 and 0.22M.

In each of these methods over 80% of the kinin precursor was lost; the preparation of plasma by dilution was therefore discontinued.

2. Dialysis

Human pseudoglobulin lost 90% of its kininogen during pressure dialysis at ½ atmosphere and 4° for 24 hours. No attempt was made to prepare whole plasma for chromatography by dialysis.

3. Gel-filtration

Dextran gel columns separated potassium phosphate from protein fractions with little loss of kininogen. They were therefore used to remove salt from plasma.

10 ml plasma was layered beneath buffer on to a column of coarse G-50 Sephadex (2.4 x 15 cm) equilibrated with 0.02 M phosphate buffer (pH 7.35, 0.10 M-NaCl) at 3 - 5°. The column was developed with the same buffer at a pressure head of 20 - 30 cm. and a flow rate of 90 ml/hour. The eluate was collected in 2.5 ml fractions. Optical density (λ275 mμ) was taken as a measure of protein concentration and specific conductivity (m.mho/cm) as a

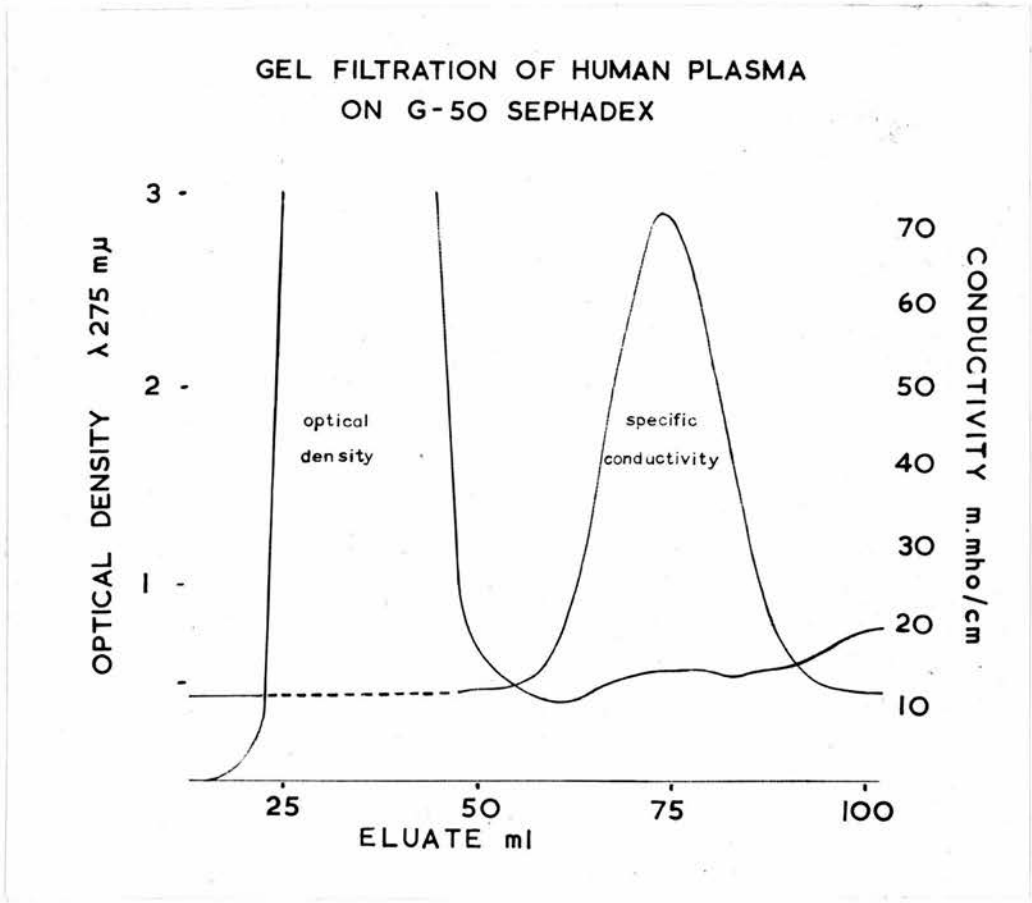

GEL FILTRATION OF HUMAN PLASMA
ON G-50 SEPHADEX
OPTICAL DENSITY λ 275 mμ
3
2
1
optical
density
specific
conductivity
70
60
50
40
30
20
10
CONDUCTIVITY m.mho/cm
25
50
75
100
ELUATE ml

FIG 1

measure of electrolyte concentration. The elution pattern is shown in Figure I ; in this experiment the salt concentration of the plasma was increased tenfold by the addition of sodium chloride. The protein peak was contained within the first 50 ml of eluate. A gap of 7.5 ml separated this from the beginning of the salt peak. The plasma was equilibrated with phosphate buffer in 35 minutes. (To avoid repetition the method described above will be referred to as "Step I").

The loss of kininogen during Step I was determined in a separate experiment. The first 50 ml of eluate was led into 200 ml ethanol. The bradykininogen content of the precipitate and of the original plasma was determined by the method of Brocklehurst and Lahiri (1962). Of the 118 units applied to the column, 98 were recovered in the eluate (about 83%). The yield was reduced to 68 units when the column length was increased to 25 cm. Free plasma kinin was extracted by ethanol from the protein peak (> 1.8 µg Bk) and from the salt peak (> 2.0 µg Bk).

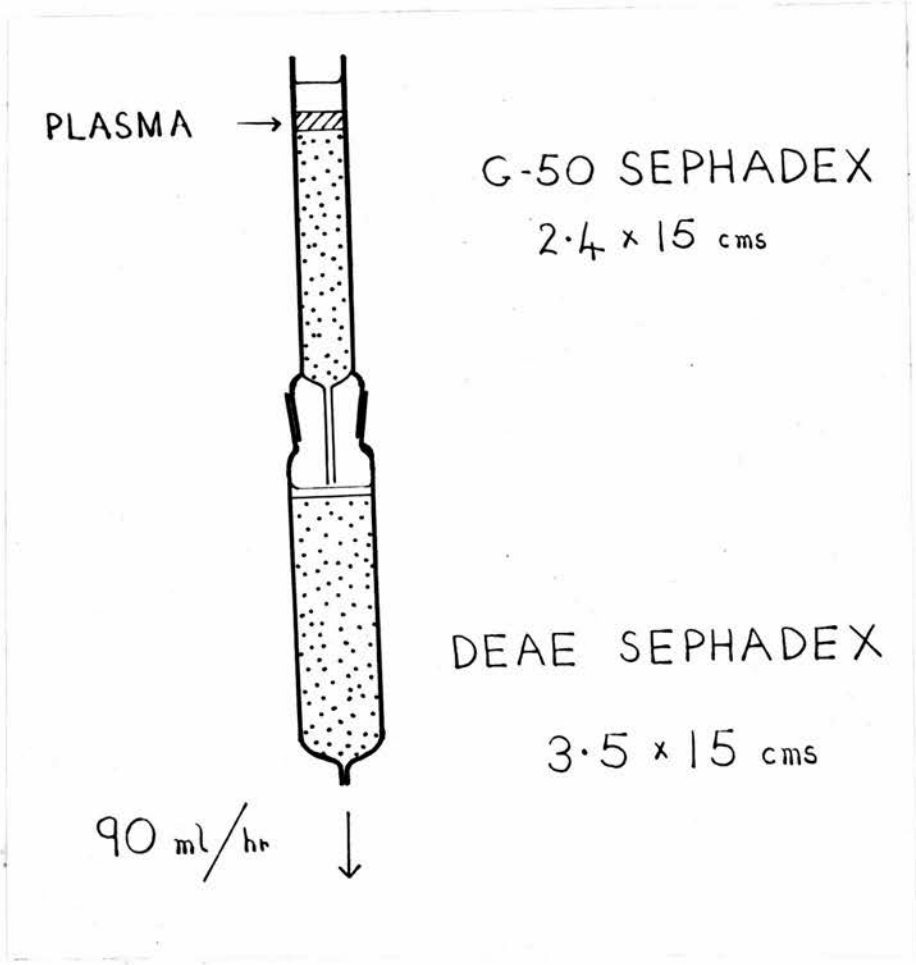

PLASMA
G-50 SEPHADEX
2·4 x 15 cms
DEAE SEPHADEX
3·5 x 15 cms
90 ml/hr

FIG 2

When 10 ml plasma was equilibrated with 0.02 M acetate buffer (pH 5.0, 0.10 M-NaCl) the yield of kininogen (27 units) was not improved. No free kinin was found in the protein peak however (< 0.07 µg Bk) and the equilibrated protein showed no further loss of kininogen during 6 hours at 4°.

The plasma kinin found in the electrolyte peak was probably formed high up on the column and separated from the protein by gel-filtration. Plasma kallikreinogen may have been activated by dilution or contact with the gel surface. The enzyme did not appear to split the substrate at pH 5.0.

Dilution and delay during gel-filtration were minimised by using the smallest adequate column for each plasma volume. The characteristics of the columns used for different volumes are given in the Appendix 10 . The rapid transfer of equilibrated plasma to the ion-exchange columns was achieved with a double column (Figure 2). The upper G-50 Sephadex column was linked to the lower DEAE-Sephadex column through a ground glass joint.

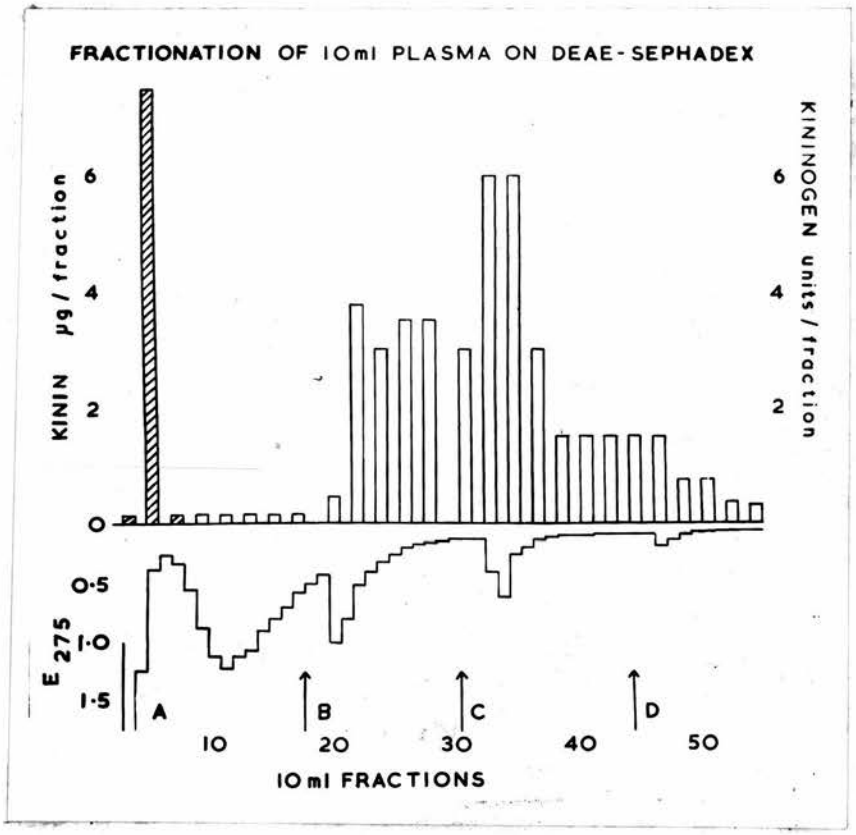

FRACTIONATION OF 10ml PLASMA ON DEAE-SEPHADEX
KININ µg/fraction
KININOGEN units/fraction
0
2
4
6
E 275
0·5
1·0
1·5
A
B
C
D
10
20
30
40
50
10ml FRACTIONS

FIG 3

CHROMATOGRAPHY ON DEAE-SEPHADEX

The eluate from Step I was led on to a column of DEAE-Sephadex A-50 (3.5 x 7.5 cm : dry weight 5.7g) equilibrated with buffer "A" (0.02 M sodium phosphate, 0.10 M sodium chloride, pH 7.35). The G-50 Sephadex column was removed immediately 50 ml of eluate had been transferred to the lower column. In this way the salt peak was prevented from interfering with the adsorption of kininogen. The plasma proteins were eluted from the DEAE-Sephadex column in four main fractions, A, B, C and D by increasing the sodium chloride concentration of the buffer.

Figure 3 is an elution diagram for this column. The arrows mark the points at which more concentrated buffers were applied. Fifty-five 20ml fractions were collected and the optical density (λ 275 mμ) of each recorded. The quantity of kinin (μg Bk) in each alternate fraction was assayed before and after incubation with trypsin (200 μg/ml, 37°, one minute)

Two optical density peaks appeared during elution with buffer A, the first representing non-adsorbed protein and the second protein in adsorption equilibrium. A band of tightly

adsorbed protein appeared as a blue zone at the top of the column. Free kinin (7.5 µg Bk) was found in fraction 5, just after the first protein peak. The concentration of kinin in fraction 5 fell rapidly at 37° and no additional kinin was released by trypsin. Fractions 9 to 17 contained negligible quantities of kinin (<0.2 µg Bk) even after incubation with trypsin.

Fractions 18 to 30 were eluted after the sodium chloride concentration had been raised to 0.13 M (buffer B). The optical density peak appeared in fractions 20 and 21 and the blue band of adsorbed protein became more diffuse but remained in the upper part of the column. No free kinin (<0.10 µg Bk) was detected in any fraction but kinin was released by trypsin from fractions 22 to 30; kininogen appeared to be in adsorption equilibrium with the cationic groups of the column at this salt concentration.

Buffer C which had a sodium chloride concentration of 0.16 M eluted a fourth optical density peak which corresponded with maximum kininogen activity. This elution coincided with the disappearance of the blue band from the top of the column. The elution of residual kininogen continued in 0.18 M sodium chloride (buffer D).

The effect of variation in plasma volume

The total amount of kininogen eluted in fraction C was measured in a series of experiments in which different volumes of plasma were applied to the DEAE-Sephadex column. In each case the equilibration with buffer A was carried out on the smallest adequate column of G-50 Sephadex (see page 66 and Appendix 10). The results are shown below.

The effect of plasma load on kininogen yield

volume of plasma	bradykininogen in fraction (units)	
(ml)	total	per ml plasma
5	15	3.0
10	38	3.8
15	30	3.0
30	30	1.0

10 ml plasma gave the highest absolute yield and was used routinely in subsequent experiments. The yield per ml plasma with a 5 ml load was only slightly inferior.

The effect of variation in column length

In later experiments the DEAE-Sephadex column was lengthened (3.5 x 15 cm: dry weight 11.5 g) and the buffer volumes were doubled.

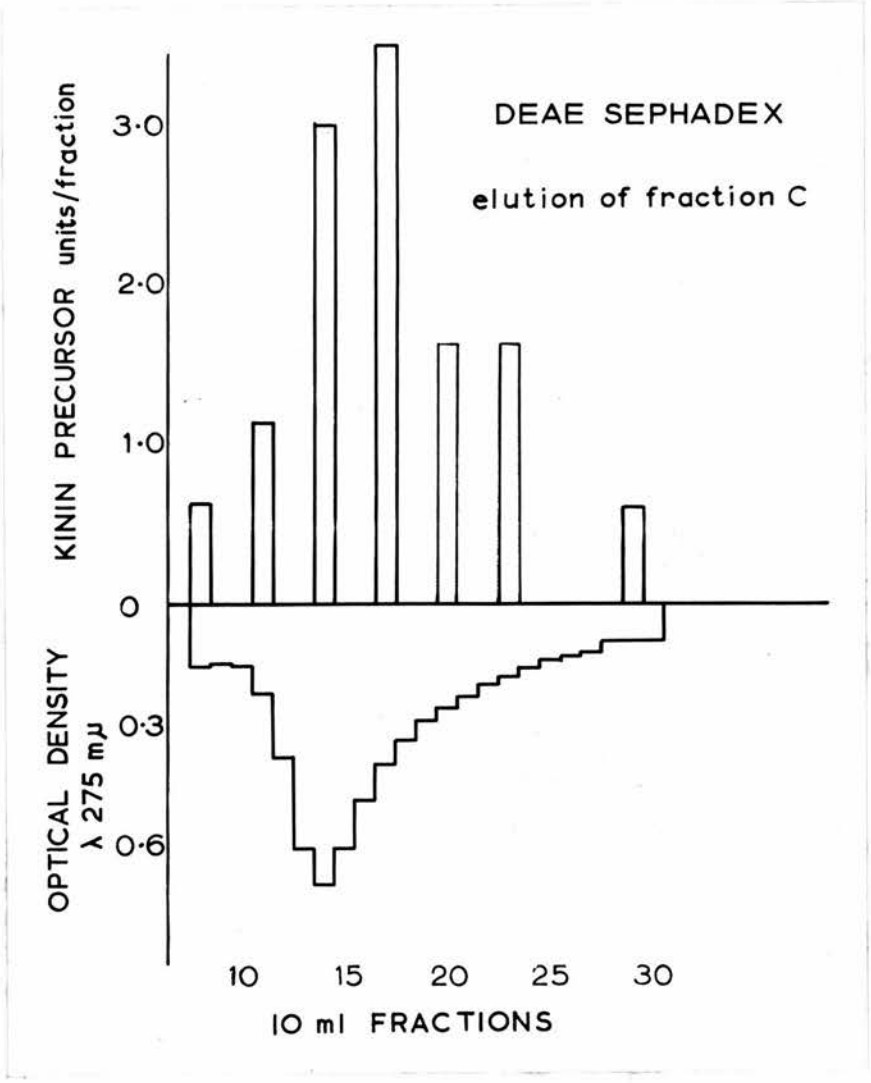

DEAE SEPHADEX
elution of fraction C
KININ PRECURSOR units/fraction
3·0
2·0
1·0
0
OPTICAL DENSITY
λ 275 mμ
0·3
0·6
10
15
20
25
30
10 ml FRACTIONS

FIG 4

This modification reduced the quantity of kininogen eluted by 0.13 M salt so that the kinin precursor was concentrated more completely in fraction C. The elution pattern for the longer column at a salt concentration of 0.16 M is shown in Figure 4. The position of the optical density peak provided a guide to the fractions containing most kinin precursor.

Table III compares the results obtained with the two column lengths. The distribution of free kinin, kininogen and protein over the four main fractions A,B,C and D is shown. Protein concentrations were estimated by the modified Folin method described in Appendix 5. The two sets of results are similar. In each case free kinin was detected only in fraction A; this disappeared during incubation at 37° and no further kinin was released by trypsin. The kininogen was found mainly in fraction C associated with about 6% of the original protein. The increase in length of the DEAE-Sephadex column improved the specific activity of fraction C from 0.91 to 1.3 µg Bk/mg protein and the yield of kininogen from 32 to 50%. The preparation of fraction C on the modified column provided "Step II" in the purification of human bradykininogen.

Table III

The fractionation of plasma on DEAE - Sephadex A-50 at 4°.
10 ml plasma = 118 units bradykininogen, 750 mg protein

Fraction	Buffer (0.02 M phosphate)		Column length = 7.5cm.				Column length = 15cm.			
	NaCl moles/l	pH	Volume ml	Kinin µg Bk	Kininogen units	Protein mg	Volume ml	Kinin µg Bk	Kininogen units	Protein mg
A	0.10	7.35	300	2.2	-	310	600	4.5	-	420
B	0.13	7.30	200	0.6	16	210	400	1.0	6.0	140
C	0.16	7.25	200	0.6	38	41	400	1.0	60	44
D	0.18	7.20	200	0.6	9.4	10	400	1.0	3.0	12

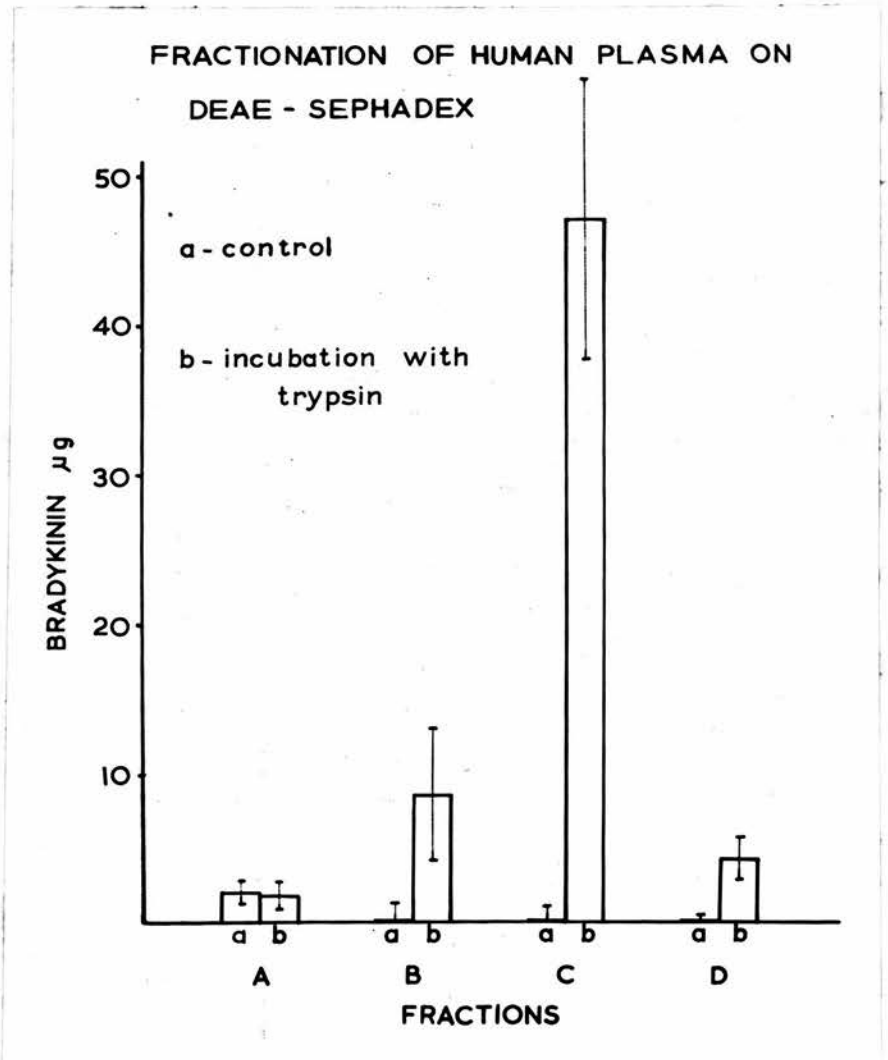

FRACTIONATION OF HUMAN PLASMA ON
DEAE - SEPHADEX
a - control
b - incubation with
trypsin
BRADYKININ µg
50
40
30
20
10
a b
a b
a b
a b
A
B
C
D
FRACTIONS

FIG 5

The reproducibility of the method was established by a series of experiments with plasma from different donors. In these experiments two measurements were made on each main fraction, (a) the kinin (µg Bk) present after 5 minutes at 37° and (b) the kinin (µg Bk) present after incubation with trypsin for 5 minutes. (100% kinin release by trypsin under these conditions required less than 5 minutes. See page 86). The full results and calculations are given in Appendix 14 : Figure 5 gives a graphical summary. Fraction A consistently contained free kinin but no kininogen. ((a) and (b) did not differ significantly). The free kinin in fractions B, C and D was always below the threshold of the biological assay (i.e. < 1.2 µg/fraction). Kininogen was regularly present in each of these fractions but notably in fraction C.

(Note. More than a dozen DEAE-Sephadex columns were run during the developmental stages of the method. The results for eight of these are tabulated in Appendix 13. Although the same qualitative pattern occurs in each experiment, the results have not been combined. Quantitative differences were due to deliberate

changes in the conditions.

The kininogen of fraction C was stable as a freeze-dried powder at 4° for several months and in solution at 37° for at least 2 hours. The further purification of this preparation of human bradykininogen is described in the next chapter.

THE PURIFICATION OF BRADYKININOGEN BY GEL-FILTRATION

INTRODUCTION

Electrophoresis in starch gel showed that fraction C contained six or more proteins. Some of these components appeared to differ widely in molecular size (page 92) and gel-filtration was therefore chosen as the method for the further purification of kininogen.

THE FRACTIONATION OF PROTEINS BY GEL-FILTRATION

The terms "gel-filtration" and "molecular sieving" are applied to methods of fractionation which depend upon the selective exclusion from solvent spaces of molecules above a critical size. Mould and Synge (1954) used collodion membranes of graded pore size to fractionate amyloses of different molecular weight. Crystalline aluminosilicates with uniform pore size have been used for the selective adsorption of small molecules (Hersh, 1961). Before similar techniques could be applied to the column chromatography of proteins it was necessary to develop an hydrophilic gel with a

uniform structure which would exclude only proteins above a critical size. Deuel and Neukom (1954) introduced a cross-linked galactomannan and Porath and Flodin (1959) the cross-linked dextrans with low water regain (G-25 and G-50 Sephadex). These provided rapid and efficient means of separating proteins from salts and small peptides but were of little direct value in protein fractionation.

The fractionation of proteins was achieved by gel-filtration on granules of 7% agar (Polson, 1961), a polymer of acrylamide and methylene bis-acrylamide (Lea and Sehon, 1962) and cross-linked dextran gels of high water regain (Gelotte, Flodin and Killander, 1962). The latter have been made available commercially as G-100 and G-200 Sephadex; they are synthesised from dextrans of selected length by reaction with epichlorohydrin. The practical and theoretical aspects of protein fractionation on these gels have been reviewed in detail by Flodin (1962). Long columns of fine particles offer high resolution when these conditions are compatible with moderate flow rates. High flow rates prevent the attainment

of equilibrium at each level on the column whereas low flow rates permit longitudinal diffusion. In each case resolution is impaired.

The behaviour of a protein during gel-filtration is determined by its molecular weight and its diffusion coefficient (Polson, 1961). There are several complicating factors however. Proteins may be adsorbed to Sephadex at low ionic strength (Glazer and Wellner, 1962), fibrous proteins may fail to penetrate the gel to the same extent as globular proteins of the same molecular weight and charged proteins within the gel may prevent the entry of molecules carrying a similar charge. Andrews and Folley (1963) calibrated dextran gel columns with proteins of known molecular weight. They found a linear relationship between the log of the molecular weight and the log. of the elution volume. This relationship held for columns of G-200 Sephadex when the molecular weights were in the range 20,000 - 500,000.

Serum proteins were resolved into three main fractions on G-200 Sephadex (Gelotte, Flodin and Killander, 1962; Flodin and Killander, 1962; Flodin, 1962). The first contained proteins of very high molecular weight which were almost totally excluded from the gel (α_2 and β_2 macroglobulins and 19S antibodies). The second consisted of an intermediate group including 7S antibodies and caeruloplasmin (mol. wt. 150,000) and the third contained several smaller proteins (albumin, mol. wt. 66,000: haemoglobin mol.wt. 67,000: transferrin mol. wt. 90,000: and α_1 glycoprotein). The pH of the buffer did not affect the elution pattern but there was a critical level for salt concentration; below 0.10 M NaCl associations between proteins impaired resolution. The bead form of the gel was used successfully in columns up to 100 cm. long but other workers have found that such columns became blocked due to the packing of the gel under its own weight.

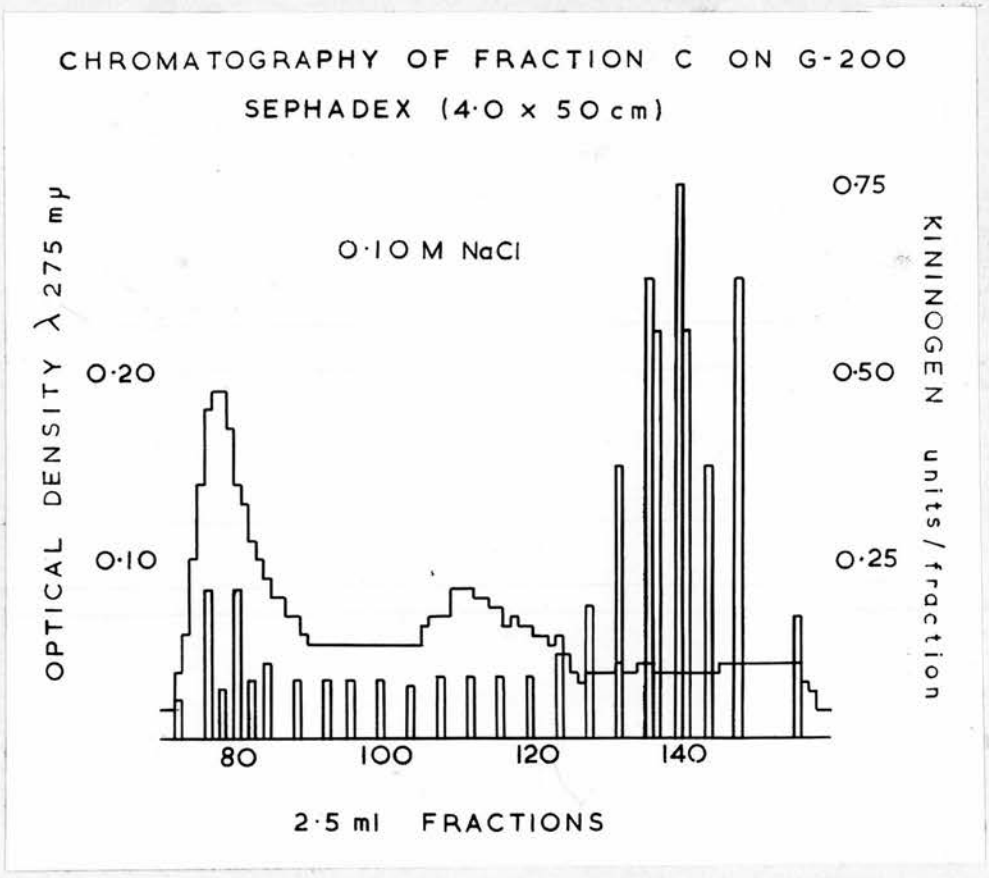

CHROMATOGRAPHY OF FRACTION C ON G-200
SEPHADEX (4·0 x 50 cm)
OPTICAL DENSITY λ 275 mμ
0·10 M NaCl
0·20
0·10
0·75
0·50
0·25
KININOGEN units / fraction
80
100
120
140
2·5 ml FRACTIONS

FIG 7

EXPERIMENTAL

THE CHROMATOGRAPHY OF FRACTION C ON G-200 SEPHADEX

Further purification of the bradykininogen in fraction C was achieved by gel-filtration on G-200 Sephadex. Fig 6 shows the elution pattern of a gel column (2.1 x 94.5 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 7.35, 0.10 M NaCl) and loaded with the freeze-dried protein and salt from 50ml fraction C dissolved in 5.0 ml of distilled water. The column was developed with 325ml phosphate buffer at a hydrostatic pressure of between 1 and 2 metres arranged to give a flow of 7.5ml per hour. Fractions of 2.5ml were collected and the optical density (λ 275 mμ) of each determined. The specific conductivity was measured in fractions 92 to 133. Alternate fractions were incubated with trypsin (60 μg/ml, 37°, 5 minutes) and the released kinin was assayed on the rat uterus. The fractions corresponding with the first half of the salt peak were assayed for free kinin.

Fractions 40 to 54 contained the main protein peak. A second flattened peak

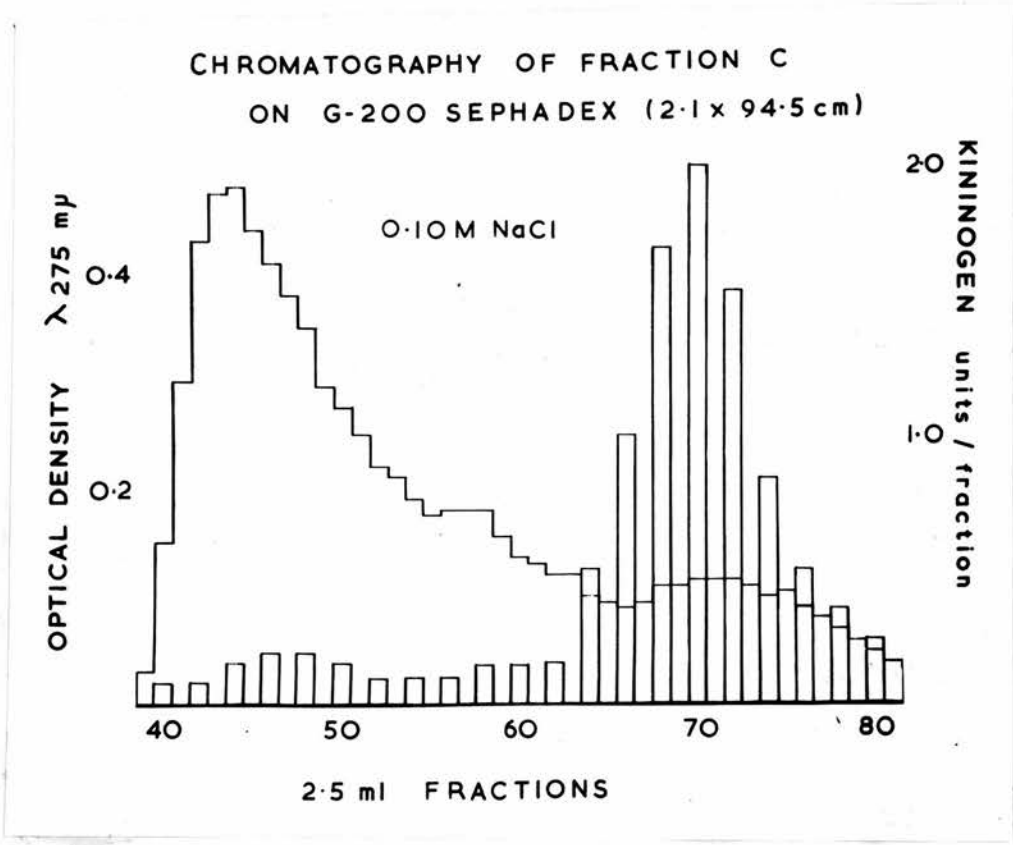

CHROMATOGRAPHY OF FRACTION C
ON G-200 SEPHADEX (2·1 x 94·5 cm)
OPTICAL DENSITY λ 275 mμ
0·4
0·2
0·10 M NaCl
KININOGEN units / fraction
2·0
1·0
40
50
60
70
80
2·5 ml FRACTIONS

FIG 6

occurred in fractions 54 to 66 and a third low peak in fractions 66 to 80. Kininogen occurred in all the fractions between 40 and 80 but the activity was concentrated mainly in fractions 64 to 76. A small increase in kininogen activity was found in 44 to 50. No free kinin was detected (i.e. less than 0.025 µg Bk/ fraction).

Gel-filtration thus separated the bulk of the kininogen from much of the other protein present in fraction C without the release of any detectable kinin during 40 hours at 18° - 20°. After three experiments on the same column the maximum flow rate had fallen to 2.5ml per hour and it was decided that shorter columns would be more suitable for repeated use.

Fig 7 shows the elution pattern obtained when the kininogen from 90ml fraction C (about 15 units) was applied to a shorter, broader column (4.0 x 50 cms). This column maintained a more suitable flow (30ml/hour) and about 80% of the kininogen was obtained in fractions 128-156. Fractions 138 and 139 pooled had a specific activity of 5.2 µg Bk/mg

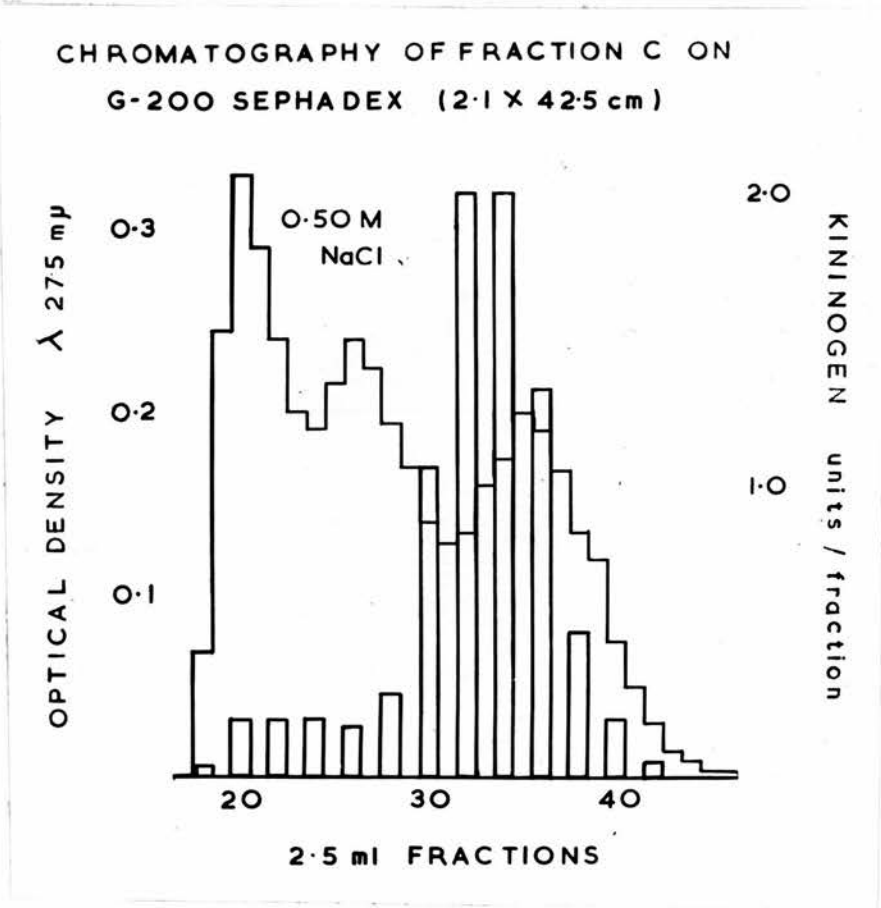

CHROMATOGRAPHY OF FRACTION C ON
G-200 SEPHADEX (2·1 X 42·5 cm)
OPTICAL DENSITY λ 275 mμ
0·3
0·2
0·1
0·50 M
NaCl
2·0
1·0
KININOGEN units / fraction
20
30
40
2·5 ml FRACTIONS

FIG 8

protein. In fractions 78, 82, 137 and 141 parotid saliva (diluted 1:10) was used instead of trypsin. The concentration of free kinin was measured in fractions 80,92,102,114,126,134, 142 and 154; none was detected (i.e. less than 0.012 µg Bk/fraction). A column of this size was mounted in a cold room at 4° and used routinely in the preparation of purified kininogen (Step III). It was large enough to receive the pooled fraction C from four DEAE-Sephadex columns run concurrently.

The influence of ionic strength

The small peak in kininogen activity during the early part of the elution was a constant finding. Gel-filtration on a column of G-200 Sephadex (2.1 x 42.5 cm) was repeated under conditions of high ionic strength (0.5M NaCl). There was little change in the elution pattern (Fig 8). The small early peak persisted under conditions in which the liklihood of protein associations was small.

Summary

The purification of human bradykininogen by Steps I,II and III is summarised in table IV ; the yield of kininogen and the specific activity of the protein at each stage are stated.

Table IV

The purification of bradykininogen

Step		bradykininogen		
		units	%	µg Bk/mg protein
	10ml plasma	118	100	0.17
I	eluate from G-50 Sephadex	98	83	-
II	fractions B,C and D	69	58	0.35
	fraction C	60	50	1.3
III	eluate from G-200 Sephadex (third peak)	4 x 12	40	5.2

THE PROPERTIES OF PURIFIED HUMAN BRADYKININOGEN

PHARMACOLOGICAL PROPERTIES

Specific Biological Activity

Fractions 69 and 71 from the column of G-200 Sephadex in the experiment illustrated in Fig. 6 were each divided into aliquots. Protein concentrations were determined by the modified Folin method using crystalline bovine serum albumin as a standard (Appendix 5) and the potential kinin was determined after incubation with trypsin (10 µg/ml) for 5 minutes. Trypsin was inactivated by heating at 100° for 10 minutes and the released kinin was assayed against synthetic bradykinin on the rat uterus using a 2+ 1 dose design (Appendix 4)

Fraction	specific activity (mean ± fiducial limits, P=0.05)
69	6.15 ± 0.90 µg/mg
71	4.89 ± 0.53 µg/mg

Although the specific activity of the purified kininogen was increased when fraction C

was submitted to gel-filtration, there appeared to be no other change in pharmacological properties.

Freedom from kinin-destroying enzymes

Samples of fraction C from two experiments were incubated with synthetic bradykinin. After 2 hours at 35° the concentration of bradykinin was compared with that of a solution of bradykinin in saline which had received the same treatment and another solution which had been stored at 0°. The results showed no evidence of kininase activity.

The stability of synthetic bradykinin in Fraction C.

t°	0.9% NaCl	Fraction C	bradykinin (ng/ml at 2 hrs)
0	+	-	12.5
35	+	-	11.1
35	-	+	11.7
35	-	+	11.1

When the incubation of trypsin (10 µg/ml) with Fraction C (600 µg/ml) was prolonged from 5 to 60 minutes, there was no evidence of destruction of the kinin which had been released. Similarly there was no loss of bradykinin-like activity when the mixture of fraction C and

parotid saliva was incubated for an additional 55 minutes.

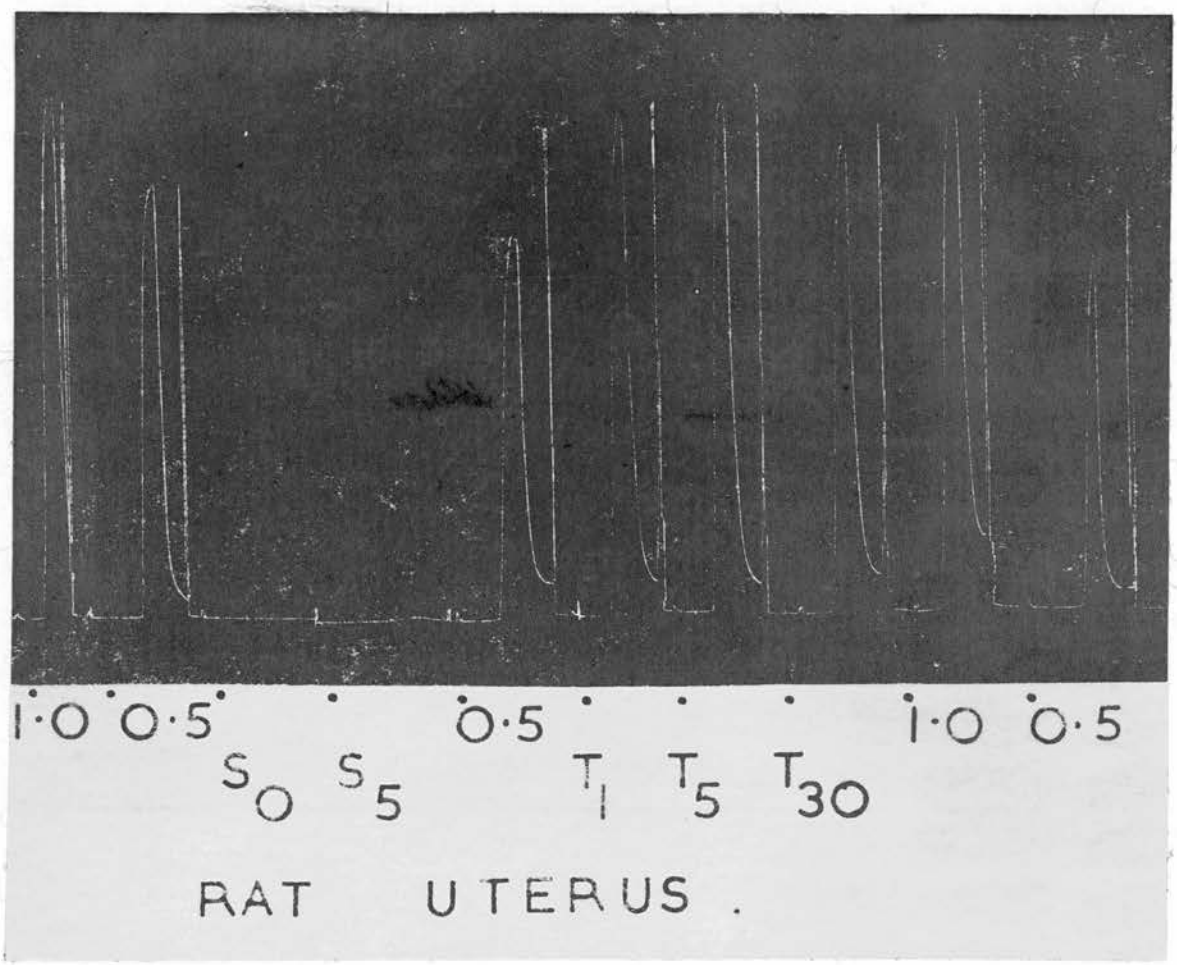
Freedom from plasma kinin

The samples of purified kininogen prepared by step II and step III were repeatedly tested for the presence of free kinin. In every case the level lay below the threshold for the biological assay; the uterus of the stilboestrol-treated rat showed no response either during contact with the sample or during the wash which followed (Fig 9). In a series of experiments the free kinin in fraction C was less than 1.0 µg Bk whilst the kinin released by trypsin was equivalent to 47 ± 9.3 µg Bk. (Fig 5 : Appendix 14).

Freedom from kinin-forming enzymes

The absence of kinin-forming enzymes from the preparations was inferred from the following observations.

A solution of fraction C which was capable of releasing 630 ng Bk/ml within 5 minutes of the addition of trypsin (10 µg/ml) contained no kinin (i.e. less than 17 ng Bk/ml) after 30 minutes at 37°.



The assay of the kinin released from fraction C by trypsin

FIG 9

0·5 ng
1·0 ng — synthetic bradykinin

$S_{0,5}$ "Substrate blanks" Fraction C incubated at 37° for 0 and 5 minutes. Undiluted Dose 0.2 ml.

$T_{1,5,30}$ Fraction C incubated with trypsin (60 µg/ml) at 37° for 1, 5 and 30 minutes. Diluted 1 : 20 Dose 0.10 ml.

Purified bradykininogen from G-200 Sephadex was exposed to acetone (20% v/v) at 18° for 4 hours; the acetone was then removed by freeze-drying and the product was incubated with fraction C for 5 minutes at 37°. No kinin was released (i.e. less than 5 ng Bk/ml). Under the same conditions parotid saliva (diluted 1 : 10) released 130 ng Bk/ml in less than one minute.

No free kinin was released at any stage in the gel-filtration of fraction C even when this occupied 40 hours at 18° - 20° (page 80).

Fraction C has been stored as a shell frozen solution at -25° for 4 to 6 months without the appearance of free kinin on thawing.

The release of kinin

Trypsin During the early part of this work trypsin was used at a concentration of 200 µg/ml for the release of kinin from fraction C and similar preparations. This followed the earlier practice of Diniz and Carvalho (1963), Lahiri (1962) and others. Later it was reduced to 60 µg/ml, and even at this level it was usually found that full release of kinin occurred during the first minute of incubation (Fig. 9).

A sample of fraction C with a protein concentration of about 600 µg/ml was incubated with trypsin (10 µg/ml) at 37°. Aliquots were transferred to a boiling water bath at 1,2,5 and 30 minutes. After heating for 10 minutes they were cooled and the kinin released was assayed against synthetic bradykinin; 100% kinin release was achieved in 2 to 5 minutes. See table V .

Parotid saliva Human saliva obtained from the parotid duct and diluted tenfold gave similar results. Full kinin release occurred in less than 5 minutes. The quantity of "bradykinin" released appeared to be greater than with trypsin. This may be due to differences in the potencies of the kinins released by the two enzymes.

Other enzymes Fraction C was incubated with a sample of the kinin-forming enzyme (10 µg/ml) purified from human plasma euglobulin by Armstrong and Mills (1963). After 30 minutes at 37° about 2.5% of the potential kinin had been released and no further release was detected at 60 minutes.

Table V

The activation of human kininogen by trypsin (10 µg/ml) and parotid saliva (diluted 1 in 10)

	bradykinin (µg/ml)			
Incubation time (min)	1	2	5	30
Fraction C + trypsin	0.23	0.44	0.63	0.63
Fraction C + parotid saliva	0.57	-	0.69	0.69
Trypsin blank	less than 0.017			
Saliva blank				
Substrate blank				

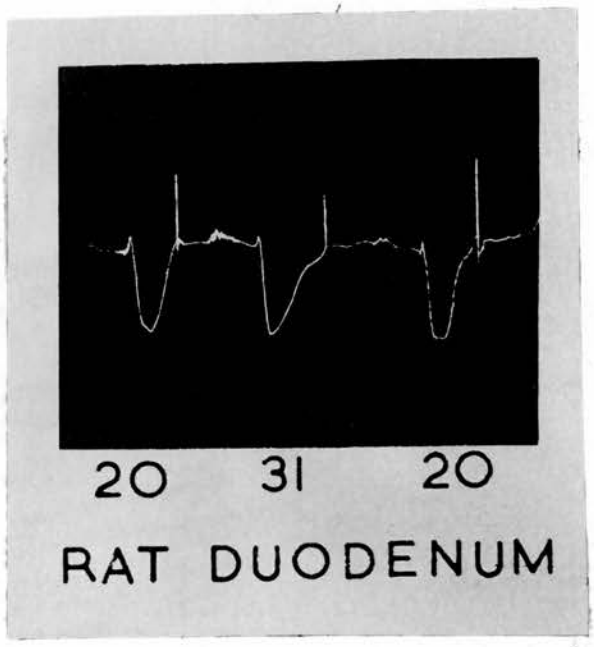


FIG 10

20 ng synthetic bradykinin

31 Fraction 31 (figure 8)
incubated with trypsin (60 µg/ml)
Diluted 1 : 4
Dose 0.10 ml.

In preliminary experiments no kinin release from the purest kininogen fractions has been observed with acetone-treated fresh plasma, diluted serum (1:20) and glass-activated plasma; in each case the plasma had been depleted of its own kininogen by prolonged incubation.

The identity of the kinin released by trypsin

The biological activity released by trypsin from fraction C and from the purified kininogen eluted from G-200 Sephadex was bradykinin-like. The rat uterus, the guinea-pig ileum and the rat duodenum (Fig 10) responded in the same way to synthetic bradykinin and to the product of kininogen and trypsin.

Fraction 31 from a G-200 Sephadex column (2.1 x 42.5 cm.) was incubated with trypsin for 15 minutes. The concentration of kinin was assayed on the rat uterus and the rat duodenum (Fig. 10).

the parallel assay of the kinin released by trypsin

assay	kinin (µg synthetic Bk/ml.)
rat uterus	0.8 -1.0
rat duodenum	0.8

The bradykinin-like activity was stable at 35° for 2 hours and at 100° for 15 minutes. The incubation of fraction C with high concentrations of trypsin (200 µg/ml) at 37° for 30 minutes (Fig. 9) resulted in small, but consistent losses of up to 10% of the total kinin.

PHYSICAL PROPERTIES

Starch gel electrophoresis

The composition of fraction C was investigated by electrophoresis in starch gel using a small scale adaptation of the original method (Smithies, 1955) (Appendix 17). Starch gel was chosen because it was known that α_2 globulins were resolved into several fractions during electrophoresis on this medium and that the protein bands would be available for elution, proteolysis and biological assay.

Samples of fraction C were concentrated twenty-fold by pressure dialysis against 0.03M borate buffer, pH 8.85, and placed in troughs cut in the gel. After passing a current of 2.0 mA for 10 hours the gel was divided longitudinally; half was stained with amido black and the other half was divided transversely into twelve ½cm strips numbered from the origin. Each strip was macerated in 3.0 ml 0.02M sodium phosphate buffer, pH 7.35 and incubated with trypsin (200 µg/ml, 37°, 15 minutes). The kinin released was assayed against synthetic bradykinin. The results are summarised in Table VI . The kininogen content of each strip is noted and where possible the six protein

bands are tentatively identified. The identification is based on published information (Smithies, 1959) and the observed behaviour of whole plasma samples run parallel to fraction C (Fig. 11). Kininogen was located in the fast α_2 region.

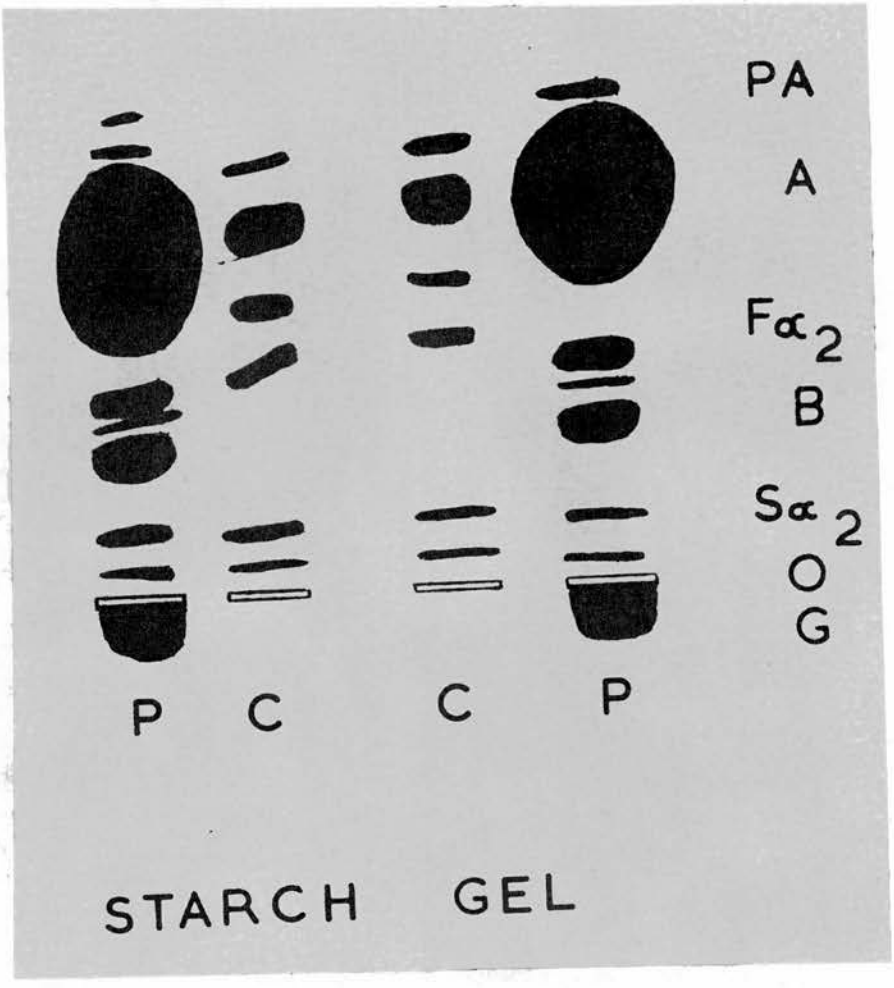


FIG II

P Plasma

C Fraction C

PA Pre-albumin

A Albumin

B β globulin

O Origin

G γ globulin

Table VI

The behaviour of fraction C during electrophoresis in starch gel

Strip (x ½cm. from origin)	kininogen (m.units/strip)	stained zones (protein / description)
1	less than 3.75	-
2	"	β lipoprotein
3	"	S α_2 (α_2 macroglobulin)
4	"	-
5	"	-
6	"	-
7	5.7	-
8	11.4	F α_2
9	5.7	post-albumin
10	less than 3.75	-
11	"	albumin
12	"	pre-albumin

Micro-immunoelectrophoresis

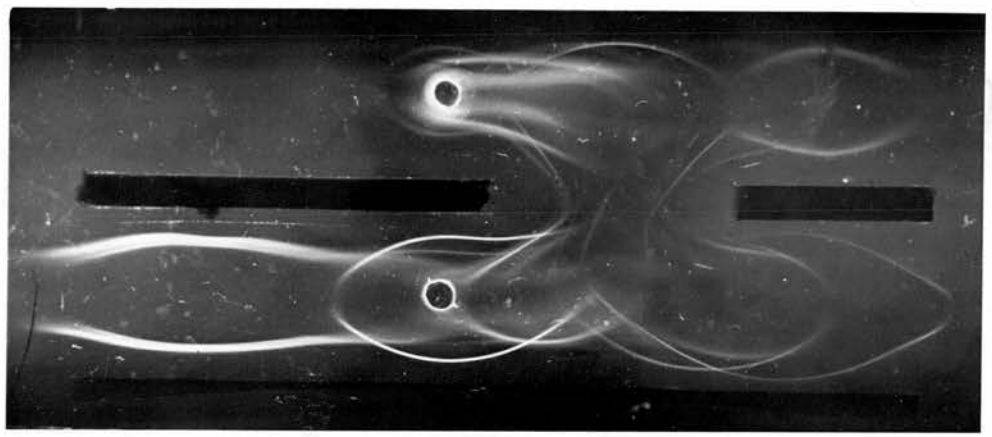
A micromethod for immunoelectrophoresis in agar gel (Scheidegger, 1955) was used to investigate the heterogeneity of the purified bradykininogen.

Fraction C and the kininogen-containing fractions from G-200 Sephadex were concentrated by dialysis followed by freeze-drying. In each case the dried protein from 25ml of eluate was dissolved in 0.05ml 0.10M veronal acetate buffer, pH 8.6 to give a protein concentration of about 50 mg/ml. These samples were compared with each other and with whole serum by immunoelectrophoresis against four anti-human sera (Institut Pasteur, 13461 and 223: Wellcome, 4505 and 4506). The results are illustrated by photographs of the slides taken under dark ground illumination between 16 and 72 hours after the addition of antiserum.

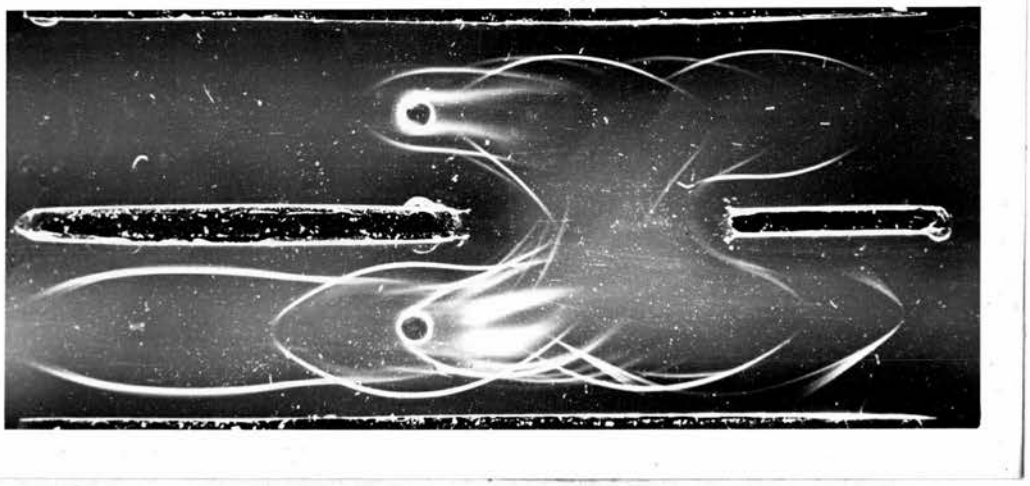
Slides 1,2 and 3 compare fraction C with whole serum against Pasteur 223. Six components are visible in slide 2 but eight or more arcs can be seen in slides 1 and 3. The fastest component of fraction C appears to be albumin. The componenets which travel slowly during electrophoresis and do not diffuse out from the

FRACTION C / PLASMA

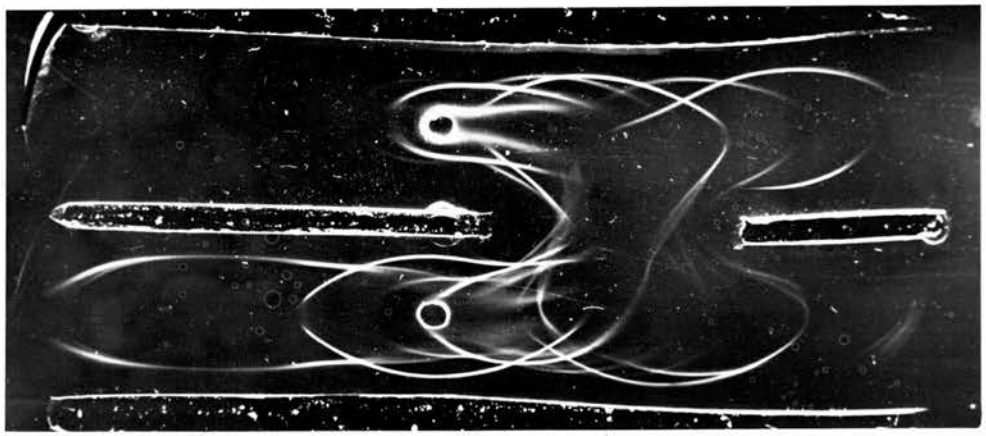
Pasteur
223



1

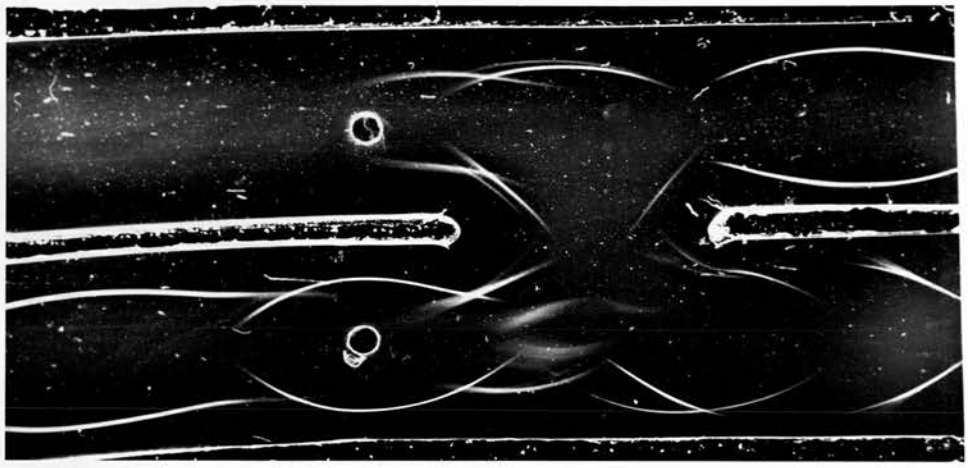


2



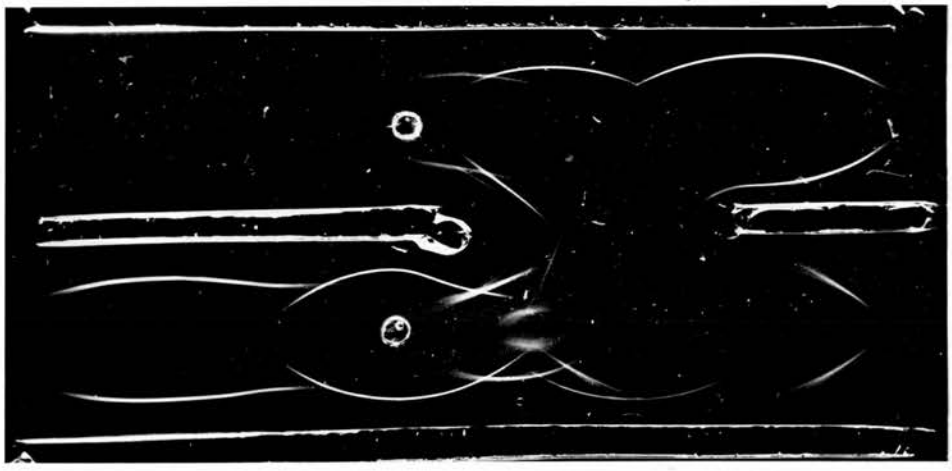
3

PURIFIED KININOGEN / PLASMA



Pasteur
223

4

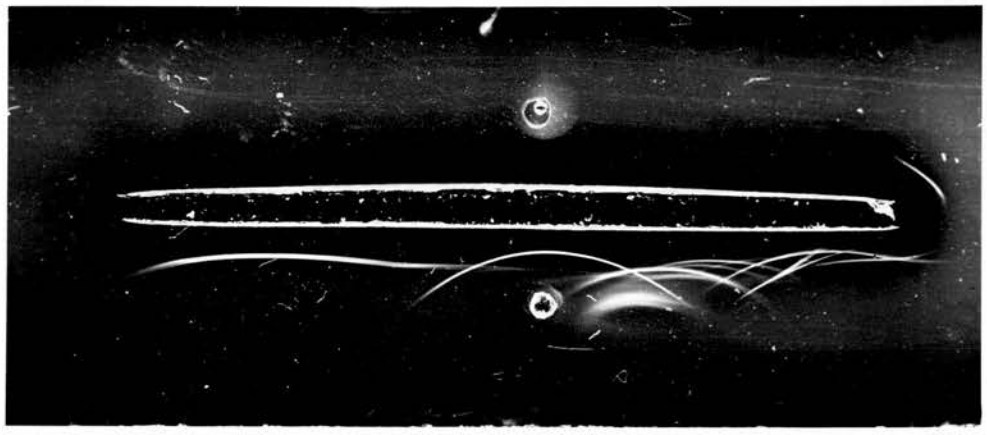


Wellcome
4505

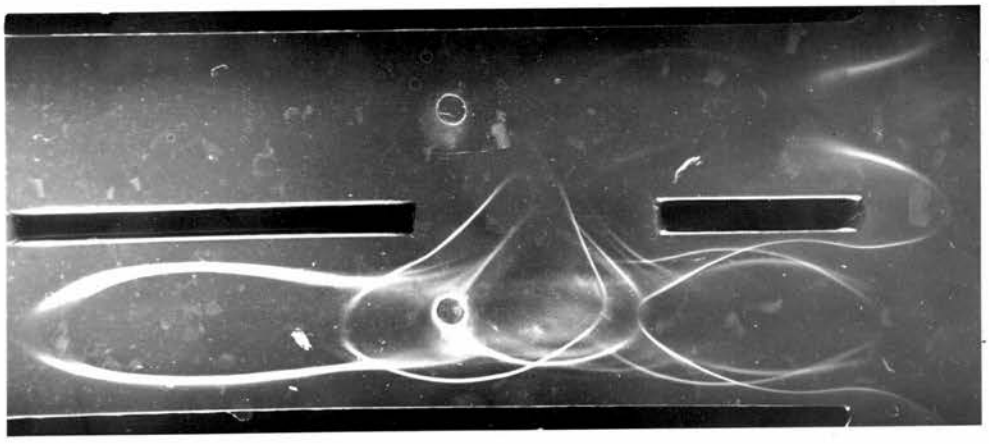
5

PURIFIED KININOGEN / PLASMA

Pasteur 223



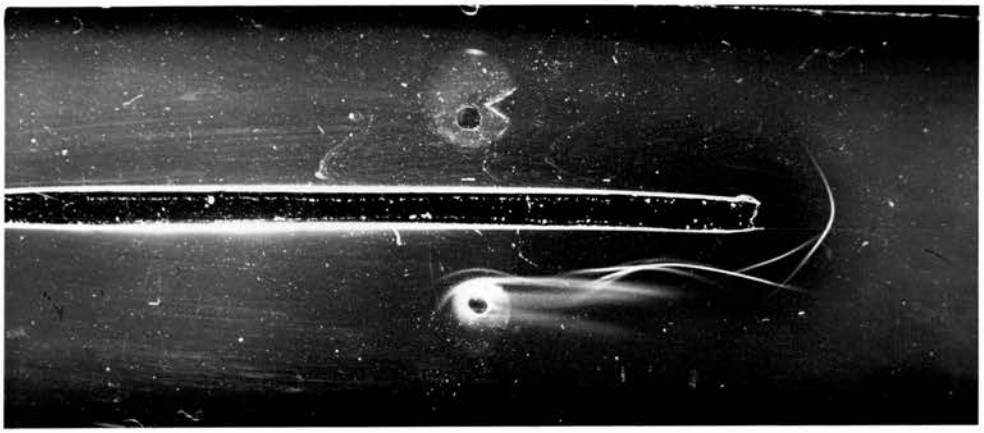
6



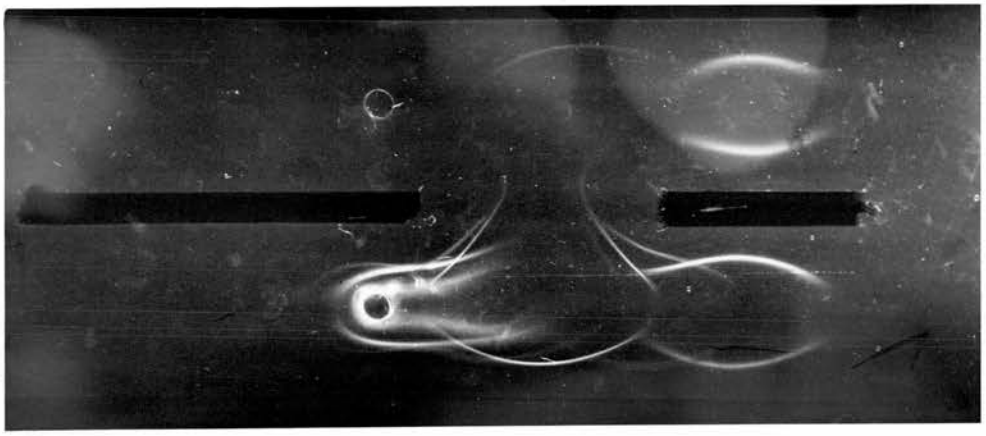
7

PURIFIED KININOGEN / FRACTION C

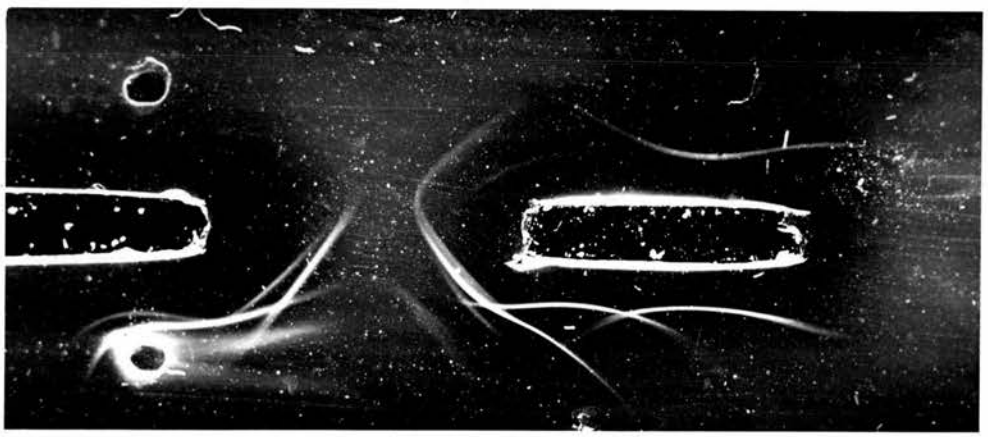
Pasteur
223



8



9



10

longitudinal axis probably correspond with the $S\alpha_2$ and β lipoprotein zones on starch gel electrophoresis.

Slides 4 and 5 compare purified bradykininogen from G-200 Sephadex (2.1 x 94.5 cm : fractions 63,65,67,73,75 and 77: Fig. 6) with whole serum against Pasteur 223 and Wellcome 4505. Four arcs are visible with each of the samples of purified kininogen; the slowest component which is absent from slides 6 and 7 may have been introduced in fraction 63, which contains the 'tail' of the preceding optical density peak.

Slides 6 and 7 compare purified bradykininogen from G-200 Sephadex (4.0 x 55 cm : fractions 127,130131,135,143,146,147,150,151 and 155: Fig. 7) with whole serum against Pasteur 223. The purified kininogen on slide 6 shows two arcs; the faster of these links with the albumin of whole serum. Slide 7 confirms the link with albumin and shows two other components.

Slides 8, 9 and 10 compare the sample of purified kininogen used in 6 and 7 with fraction C against Pasteur 223. On slide 8 a continuous line links the albumin components of fraction C and the more highly purified kininogen. Slide 9

shows cross-over between the slower component of the purified kininogen and a protein in fraction C. Slide 10 shows three proteins in the purified fraction, the slowest linking with an arc in fraction C.

The most highly purified sample of bradykininogen therefore contains at least three proteins. One of these is albumin which was shown to have no kininogen activity in the starch gel experiments. The remaining two proteins behave as α_2 globulins on immunoelectrophoresis but they have not been identified more precisely.

Gel-filtration

Rabbit γ globulin, human kininogen, bovine serum albumin and crystalline ovalbumin were compared during gel-filtration on a column of G-200 Sephadex (2.1 x 42.5cm) equilibrated with 0.50M NaCl buffered at pH 7.1. In each experiment the protein (5 - 12 mg) was dissolved in 5.0 ml of buffer which was at least 1.0 M with respect to NaCl, and layered on to the top of the column. The positions of the optical density peak (λ 275mμ) (or the biological activity peak in the case of kininogen) and the specific conductivity peak were determined. The table VII below gives the volumes of buffer required for the elution of half the protein peak and half the conductivity peak. The variability of the latter is a measure of the failure to achieve complete standardisation of the conditions. The values for the molecular weight of the standard proteins are taken from Edsall (1953).

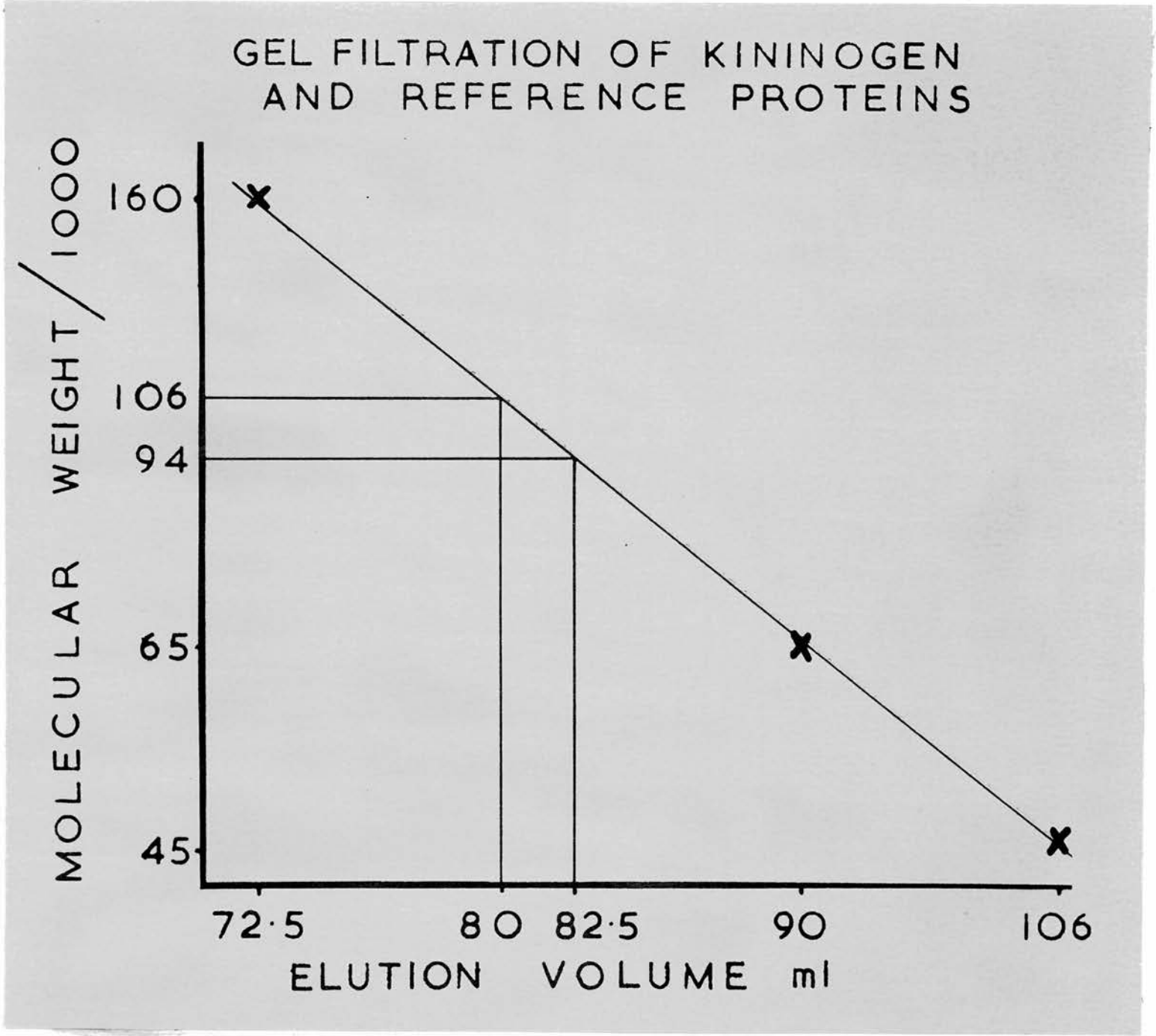

GEL FILTRATION OF KININOGEN
AND REFERENCE PROTEINS
MOLECULAR WEIGHT / 1000
160
106
94
65
45
72·5
80
82·5
90
106
ELUTION VOLUME ml

FIG 12

Table VII

The elution of human kininogen and other proteins from G-200 Sephadex

buffer=0.02M sodium phosphate, pH7.10, 0.50M NaCl

protein (mol. weight)	protein elution vol. (ml)	salt elution vol. (ml)
rabbit γ globulin (160,000)	72.5	155
kininogen (donor 1)	82.5	151
kininogen (donor 2)	80.0	148
bovine albumin (65,000)	90.5	155
ovalbumin (44,000)	106.0	159

Figure 12 shows the curve obtained by plotting log. elution volume against log. molecular weight (Andrews and Folley, 1963). The elution volumes for kininogen from donors 1 and 2 correspond with molecular weights of 106,000 and 94,000 . (Donor 2 was of haptoglobin type 2-2). The validity of these estimates is criticised in the discussion.

DISCUSSION

Development of a suitable purification procedure

The protein precursor of the plasma kinins is not detectable; its presence can only be inferred in retrospect after the biologically active peptides have been released by a suitable enzyme. The enzyme chosen for this purpose was trypsin. Partially purified trypsin was available commercially and it was known to release bradykinin from pseudoglobulin, methionyllysylbradykinin and lysylbradykinin. It was assumed that any protein precursor of the three characterised kinins would give bradykinin on incubation with trypsin. The precursors of kinins E,F and S (Armstrong and Mills, 1963) may exist as distinct proteins which do not yield their kinins with trypsin. If this is the case the present investigation is not relevent to these kininogens.

The kinin-forming pro-enzymes of plasma remain inactive under a very narrow range of physical conditions. Immediately a fractionation procedure is embarked upon, the plasma bradykininogen is exposed to kinin-forming

enzymes and it suffers progressive depletion by kinin release. There are two ways of avoiding this,

i. inactivation of the kinin-forming enzyme as it is formed, and
ii. fractionation under physical conditions which prevent enzymic activity.

Plasma kallikrein is inhibited by DFP, soy bean trypsin inhibitor and pancreatic trypsin inhibitor. Each might be used to protect kininogen during the early stages of purification. DFP does not prevent the subsequent activation of kallikreinogen. It would therefore be necessary to activate all the proenzyme in the presence of DFP. The loss of kininogen during this process might be considerable. There is another objection to DFP. It may combine with groups in the kininogen molecule, serine units for example, and alter the vulnerability of the protein to physiological kinin-forming enzymes. Since the main aim behind this work was the preparation and identification of a physiological substrate for kinin-forming enzymes, any avoidable alteration in the protein molecule was

unacceptable. SBTI and PTI may have been effective in reducing losses of kininogen but if any trace remained in the final product, it could seriously interfere with later enzyme studies.

It seemed wiser to accept the presence of kinin-forming enzyme as inevitable during the early stages and to operate under physical conditions which were extreme enough to discourage enzymic activity. Fractionation with ethanol at -5° was a reasonable first choice but facilities for low temperature work were not available and the Cohn fractions available had been prepared from outdated, kininogen-depleted plasma. The results of Habermann (1963) and his co-workers now suggest that further exploitation of the Cohn methods would have been profitable but at the time fractional extraction with ammonium sulphate solutions at 4° seemed a practical alternative. Bradykininogen was known to be stable as an ammonium sulphate precipitate and it was hoped that suitable conditions might be found for the extraction of kininogen and kinin-forming enzyme in separate fractions. The resolution was inadequate however and no kininogen-rich

fraction was kallikrein-free. Potassium phosphate buffers offered better control of the conditions which determined precipitation, and adequate resolution was expected. Narrow precipitation limits were established but big losses of kininogen were still suffered.

The established methods for the inactivation of interfering enzymes in plasma were deliberately avoided because they seemed to carry a serious risk of denaturation. There was no advantage in obtaining a high yield of kininogen if its characteristics had been altered in the process.

The rapid equilibration of plasma with acetate buffer at pH 5.0 was an attempt to establish conditions which were unfavourable to the kinin-forming enzymes of plasma but which carried only a small risk of denaturation. The kininogen in the eluate seemed stable but the losses during the early stages of gel filtration were discouraging. At this stage it was decided to use an ion exchange method of fractionation as the first purification step hoping that it might be possible to separate kinin-forming enzymes from kininogen before

serious losses had been sustained. After the ionic strength of plasma had been lowered the kinin precursor became tightly adsorbed to DEAE-Sephadex. Large volumes of buffer could be passed through the column and the bulk of the unwanted protein eluted. The kininogen remained firmly bound until the salt concentration of the buffer was increased. When this was done the kininogen was eluted free from kinin-forming enzymes and from free kinin. The conditions were adjusted until the best yields were obtained and an attempt was made to increase the scale of the method. When larger volumes of plasma were applied to the column, the yield of kininogen was reduced. This was attributed to the passing of kinin-forming enzymes over the adsorbed kininogen for a longer time.

The combination of Steps I and II gave 50% of the original kininogen in fraction C. One may argue that the half lost was different from the half recovered in being more vulnerable to the kinin-forming enzymes. A recovery experiment with labelled kininogen would be necessary to exclude selective loss. We know that small changes in the method reduced the

yield sharply. Increasing the length of the G-50 Sephadex column reduced the yield from 50% to 15%, and doubling the plasma load had a similar effect. We can therefore state that the material we normally recover is very readily attacked on the columns, and reasonably assume that the unavoidable losses occur in this way.

It has been implied above that the losses of kininogen are the result of enzymic action and not caused by the physical conditions used. This assumption is supported by later experience with fraction C. The kininogen in this fraction is very stable. The losses during storage and during prolonged chromatographic separations on G-200 Sephadex are small.

An assessment of the method

At the beginning of this thesis the criteria of a satisfactory method were described. The extent to which these have been realised will now be considered.

1. "The purified bradykinin precursor shall be free from kinin-forming enzymes and antagonists of these enzymes and also from kinin-destroying enzymes."

Fraction C and the purified kininogen from G-200 Sephadex are very vulnerable to trypsin and to the kallikrein of parotid saliva. Yet there is no release of kinin when these preparations are incubated alone at 37° for 2 hours. The preparations do not contain enzymes like trypsin or salivary kallikrein. They could however contain inactive kallikreinogen or a kallikrein which lacked an essential cofactor. Attempts to demonstrate the former by acetone activation failed, but the second possibility is not excluded.

Trypsin and parotid saliva release the potential kinin from fraction C and from the kininogen eluted from G-200 Sephadex very rapidly even when the enzyme is highly diluted. This suggests that the preparations are free from enzyme antagonists but there is no quantitative evidence to support this suggestion.

The purest preparations of kininogen do not increase the small loss of bradykinin sustained during 2 hours at 37°. They are kininase-free.

2. "Conditions which carry a risk of denaturation shall be avoided."

The risk of denaturation during the preparation of fraction C is minimal. The low temperature (3 - 5°) and the near physiological buffer systems (pH 7.35 - 7.20; 0.10 - 0.16 M-NaCl) favour the preservation of the native state. The most hazardous step is probably the solution of the freeze-dried protein and buffer salts prior to gel filtration on G-200 Sephadex.

3. "The purified protein shall retain its biological activity under convenient storage conditions for long periods."

Experience with the most highly purified fractions is still limited. Fraction C has however been kept as a shell-frozen solution and as a freeze-dried powder for six months without serious loss of activity.

4. "A high proportion of the kinin precursor present in fresh plasma shall be recovered."

When 10 ml plasma are passed through steps I, II and III, about 40% of the original

kininogen is obtained in the final product (table IV). This is not good by absolute standards but it compares favourably with the other methods available for the purification of plasma kininogen. Henriques et al (1962) recovered 34% of the kininogen present in acid-treated plasma but this figure takes no account of the losses which accompanied acid treatment; Greenbaum and Hosoda (1963) recovered less than 3% of the kininogen present in the crude globulin precipitate; Webster and Pierce (1963) obtained 15% of the kininogen present in outdated human plasma and Habermann and Rosenbusch (1962) do not quote the final yield.

5. "The quantity of the purified kininogen shall be adequate for the detailed study of the activation by several enzyme systems and for the preliminary characterisation of the protein."

10 ml plasma yield about 10mg protein which represents about 50 units of kininogen. The sensitivity of the rat uterus is so great that this corresponds with about 50,000 detectable doses of bradykinin. The kininogen from 10 ml plasma is therefore adequate for several activation experiments. The small quantity of

protein limits the biochemical investigations which are practicable.

6. "The final product shall be homogeneous as judged by a) protein fractionation procedures with a high degree of resolution and b) measurement of specific biological activity".

The final product is not homogeneous. It contains albumin and at least two α_2 globulins. It is possible that neither of these is the bradykinin precursor. The antisera used in the immunoelectrophoresis were evoked by human sera which probably contained no intact kininogen. The antisera may lack antibodies to human bradykininogen if the loss of kinin alters the antigenicity of the parent molecule.

The specific activity of the most highly purified samples of kininogen is about 6 µg/mg. This compares favourably with the values obtained for other species by other groups. It is inferior however to the values obtained by Habermann and Rosenbusch (1963) for purified ox kininogen.

reference	specific activity of purified kininogen (µg Bk/mg of protein)
Henriques et al (1962)	3.14 (trypsin) 5.0 (snake venom)
Habermann and Rosenbusch (1962)	10 - 20
Greenbaum and Hosoda (1963)	0.8 - 1.0
Webster and Pierce (1963)	not given

If it is assumed that the molecular weight of the human bradykinin precursor is about 100,000 and that one molecule of bradykinin (mol. wt. 1131) is released per molecule of precursor, it follows that 6 µg bradykinin are derived from 600 µg protein and that 40% of the purified protein is not kininogen.

The physical properties of human bradykininogen

During electrophoresis in starch gel the human bradykinin precursor migrated as a fast α_2 globulin. This suggested that its molecular weight was of the same order as caeruloplasmin (mol. wt. 150,000). The estimates of the molecular weight from the gel filtration experiments were 94,000 and 106,000 (figure 12). The behaviour of proteins during gel filtration is however influenced by factors other than molecular weight. These may lead either to under estimation or to over estimation.

Adsorption of kininogen to G-200 Sephadex would lead to under estimation of its molecular weight. The relevant experiments were however carried out in 0.5M NaCl which makes adsorption very unlikely. Premature elution leading to exaggeration of the molecular weight could be produced by association with other proteins, deviation from a globular form or by the presence of a large charge on the molecule. The high salt concentration makes association unlikely but the other factors can not be excluded. The molecular weight probably lies between 160,000 and 65,000 but less confidence can be placed in the lower limit.

Is bradykininogen a known α_2 globulin?

A number of proteins including bradykininogen migrate as α_2 globulins during paper electrophoresis. Several of these are listed below. Bradykininogen may be an unknown protein or it may have been described in a different context by workers who were concerned with other properties and did not investigate the peptides released by trypsin. There is a third possibility; the kinin-depleted molecule may have been isolated independently and given another name. This section deals with the pertinent characteristics of several α_2 globulins and examines the possibility that each may be bradykininogen.

α_2 lipoprotein (β lipoprotein, lipoeuglobulin). This protein which has a molecular weight of about one million migrates very slowly in starch gel (Smithies, 1959). It is found in Cohn fraction III-0 and is most unlikely to be the kinin precursor. The component of fraction C which behaved like β lipoprotein in starch gel had no biological activity (figure 11, table VI).

α_2 macroglobulin. This high molecular weight α_2 glycoprotein was eluted in the first peak from G-200 Sephadex (Flodin and Killander, 1962) and migrated to the $S\alpha_2$ position in starch gel (Smithies, 1959). This contrasts markedly with the described behaviour of bradykininogen.

caeruloplasmin (copper combining globulin). This protein migrates to the $F\alpha_2$ position in starch (Smithies, 1959) but is eluted from G-200 Sephadex before the third peak (Flodin and Killander, 1962). The blue zone on the DEAE- Sephadex column ceased to be visible under the conditions which achieved the elution of bradykininogen (page 68). Had the two been identical however bradykininogen would have been eluted from G-200 Sephadex in the same fractions as rabbit gamma globulin (mol. wt. 160,000).

haptoglobin. The haptoglobin in homozygous subjects (1,1) closely resembles bradykininogen; it migrates as a fast α_2 globulin in starch (Smithies, 1959), has a molecular weight of 85,000 or 100,000 and is found in Cohn fraction IV (Herman - Boussier, Cloarec and

Cheftel, 1962). A similar protein is present in the serum of heterozygous subjects (2,1) in association with several different haptoglobins. Homozygous subjects (2,2) however do not have a haptoglobin with these properties. Their haptoglobins behave like larger molecules.

Bradykininogen prepared from a homozygous subject (2,2) was eluted from G-200 Sephadex in the same position as the kinin precursor from a donor of one of the other types (2,1) (table VII). It is unlikely that bradykininogen is a haptoglobin.

α_{2z} globulin. This glycoprotein contains 10% sugar and forms soluble complexes with zinc (Heremans, 1961). The description closely resembles that of protein H isolated by Steinbuch and Loeb (1961). The published data about these proteins are not adequate for a comparison with the known properties of human bradykininogen.

Are there two kininogens?

The behaviour of fraction C on G-200 Sephadex is shown in figures 6,7 and 8. In

each case a small peak in kininogen activity coincides approximately with the first optical density peak. The early kininogen peak is more prominent in the columns eluted with buffer of low ionic strength (0.10M NaCl) but is still present when 0.50 M NaCl is used.

The apparent separation of kininogen into two components may be due to the association of part with a larger protein or to the presence of a second kininogen, perhaps a polymer of the basic unit. The persistence of the early peak in the presence of 0.5M NaCl suggests that an association phenomenon is not responsible. No other investigation provided definite evidence for the existence of two different kininogens in human plasma.

Purified bradykininogen as a substrate for kinin-forming enzymes

The purified kininogen eluted from G-200 Sephadex released its potential kinin rapidly in the presence of low concentrations of trypsin or parotid saliva. The release of kinin by trypsin was not prevented by 0.5M NaCl. (0.20 M NaCl produced about 50% reduction in the activity of trypsin on lactalbumin, Yon 1960).

There was however no release of kinin by human plasma or serum after a series of treatments which are said to activate plasma kallikreinogen. Only a few experiments of this type were carried out and the significance of the results is uncertain.

It is possible that the substrate for plasma kallikrein is a labile kininogen which has been lost during the purification procedure but the results of Webster and Pierce (1963) make this unlikely. The purified human kininogen which they prepared released bradykinin when incubated with a purified plasma kallikrein. This kininogen represented less than 15% of the kininogen present in outdated plasma.

The failure of treated plasma to release kinin from purified bradykininogen may be due to the presence of an antagonist in the plasma or to the depletion of an essential cofactor like the factor B described by Keele (1960).

The kinin-forming enzyme obtained from human plasma euglobulin by Armstrong and Mills (1963) was incubated with fraction C. Less

than 2.5% of the potential kinin was released. This enzyme was shown by Armstrong and Mills to release not bradykinin but kinin E from intact plasma. The kinin released by fraction C may represent contamination of bradykininogen with a small quantity of "pro-kinin E".

When trypsin acts on the purified human bradykininogen the kinin released has the pharmacological properties of bradykinin. The results obtained by Webster and Pierce (1963) suggest that it is bradykinin but parallel bioassays, paper chromatography and electrophoresis will be necessary before the identity is established.

The study of kinin forming enzymes under controlled conditions requires a substrate which is free from kinin and the plasma enzymes which release and inactivate kinin. If such studies are to be relevant to the in vivo state the substrate must resemble closely the kininogen of circulating plasma i.e. the native state of the protein must be preserved and the yield of kininogen must be high. The method

described in this thesis comes closer to satisfying these requirements than the published methods. It is hoped that the purified human bradykininogen so obtained will prove useful in the investigation of kinin release in physiological and pathological states.

SUMMARY

1. Unsuccessful attempts were made to purify the bradykininogen* from human plasma by fractional extraction with ammonium sulphate solutions of decreasing ionic strength. Fractionational precipitation with potassium phosphate buffers at pH 5.8 also proved unsatisfactory. The heavy losses of bradykininogen during these procedures were attributed to the action of plasma kallikrein.

2. Plasma was prepared for chromatography by gel filtration on G-50 Sephadex (Step I) without preliminary fractionation. Bradykininogen became firmly adsorbed to DEAE-Sephadex A 50 at a sodium chloride concentration of 0.10M. A stable, bradykininogen enriched fraction ("fraction C") was eluted by stepwise increase in sodium chloride concentration (Step II).

3. A highly purified preparation of human bradykininogen was obtained from fraction C by gel filtration on G-200 Sephadex (Step III).

4. The yield of bradykininogen and the specific activity * of the protein fractions at each stage in the purification procedure are summarised in table IV (page 81). The yield was higher than that obtained with any published method for the purification of bradykininogen from plasma. The specific activity of the final product was lower than that reported for ox bradykininogen by Habermann and Rosenbusch (1962) but higher than the values obtained by other workers. An important advantage in this method is the absence of steps which carry a risk of denaturation.

5. Human bradykininogen migrated as a fast α_2 globulin during electrophoresis in starch gel. Comparison with reference proteins on columns of G-200 Sephadex indicated a molecular weight of 100,000. Micro-immunoelectrophoresis detected albumin and two α_2 globulins in the purest preparations.

6. The purified bradykininogen was shown to be free from kininase, plasma kinin and active kinin forming enzymes. The "potential bradykinin"* was released rapidly by low

concentrations of trypsin and by parotid saliva. Acetone treated plasma and a purified kinin forming enzyme from human plasma euglobulin did not release the potential bradykinin.

7. Purified human bradykininogen may prove a useful substrate for the study of kinin forming enzymes under the near physiological conditions and for the detailed investigation of the kinin forming system in human plasma.

* A glossary of terms which require definition faces page one.

APPENDICES

GENERAL

1. collection of venous blood
2. collection of parotid saliva
3. estimation of plasma kininogen and free kinin
4. specimen of 2 + 1 dose assay
5. measurement of protein concentration
6. measurement of specific conductivity

FRACTIONAL PRECIPITATION AND EXTRACTION

7. fractional extraction with ammonium sulphate
8. potassium phosphate buffers

GEL FILTRATION OF PLASMA

9. G-50 Sephadex : preparation, packing and regeneration
10. column dimensions and characteristics

ANION EXCHANGE CHROMATOGRAPHY

11. sodium phosphate buffers
12. DEAE- Sephadex : preparation, packing and regeneration
13. fractionation of plasma : summary of 8 experiments

APPENDICES

GENERAL

1. Collection of venous blood

materials

Inj. heparin B.P. 5,000 i.u./ml. (Evans Medical Ltd. Liverpool)

2% dimethyldichlorosilane in carbon tetrachloride. (silicone "repelcote", Hopkins and Williams).

method

Blood was withdrawn from the antecubital vein through a wide bore needle and polythene tube, into a siliconed, stoppered centrifuge tube graduated at 25 ml. Rapid dispersion of the anticoagulant (500 i.u. heparin in 0.5 ml. 0.9% NaCl) was ensured by repeated inversion of the tube. After spinning the tubes at 2,000 g and 3 - 5° for 15 minutes in a refrigerated centrifuge 10 ml. plasma were withdrawn through a polythene tube into a siliconed syringe lubricated with light paraffin.

2. Collection of parotid saliva

Uncontaminated parotid saliva was collected through a fine polythene catheter which drained a perspex cup placed over the ampulla of the parotid duct. The cup was held securely against the buccal mucosa by applying gentle suction to an annular trough cut around the cup in the perspex block. Salivation was stimulated by applying citric acid and sucrose to the back of the tongue.

3. Estimation of plasma kininogen and free kinin materials

Synthetic bradykinin (Sandoz, BRS 640 batch no. 0412).
lyophilized crystalline trypsin (Tryptar; Armour Pharmaceutical Co. Ltd., Eastbourne, England)
atropine sulphate
mepyramine maleate
ethanol (distilled in glass over anhydrous calcium chloride).
stilboestrol in arachis oil (100 µg/ml.)

Estimation of kininogen in whole plasma

0.2 ml. aliquots of plasma were ejected rapidly from a blow out pipette into 5 ml. 80% ethanol at 0°. The suspended precipitate was heated rapidly to 80-90° and held at that temperature for 20 minutes. The suspension was centrifuged and the ethanol removed by decantation. After washing in distilled water the precipitate was suspended in 0.02 M sodium phosphate buffer, pH 7.35, 0.10 M - NaCl. Trypsin was added to give a final concentration of 200 µg/ml. and the agitated suspension incubated at 37° for 20 minutes. The enzyme was then inactivated by heating at 100° for 10 minutes. The samples were then frozen or assayed immediately.

Estimation of kininogen after gel filtration

The 50 ml. eluate from the G-50 Sephadex column was allowed to run directly into 200 ml. absolute ethanol. The protein suspension was mixed vigorously and 5 ml. aliquots were withdrawn rapidly. Each was treated exactly as described above.

Estimation of kininogen in purified samples

Protein fractions eluted from the DEAE- and G-200 Sephadex columns were incubated with trypsin (60 or 10 µg/ml.) for 5 minutes unless otherwise stated. No denaturation in ethanol was carried out. The fractions produced by the precipitation and extraction methods were however treated like whole plasma.

The estimation of free kinin

Aliquots were heated to 100° for 10 minutes immediately after collection in order to denature enzymes which might have been able to release or inactivate kinin. In a few experiments kinin was extracted in hot ethanol. The ethanol was removed at 40° using a vacuum pump and capillary leak.

Biological assay

The kinin released from whole plasma and from the precipitation fractions was assayed on the guinea pig ileum. The greater sensitivity of the rat uterus was essential for the examination of fractions from the chromatographic experiments. The rat duodenum was used to confirm that the kinin released from the purified kininogen was bradykinin-like. It was not used for routine assay.

Guinea pig ileum

A piece of terminal guinea pig ileum about 2 cm. long was suspended in a 2ml. bath at 34° to 36°. The tissue was bathed in oxygenated Tyrode solution. A frontal writing lever with a six fold magnification exerted a tension of 0.5 g on the tissue.

45 seconds contact between drug and tissue, every 4 minutes was usually satisfactory. Mepyramine maleate and atropine sulphate (1 µg/ml) were used to increase specificity.

Rat uterus

Virgin, albino rats (100-150g) were given a subcutaneous injection of 10 - 15 µg

stilboestrol in arachis oil. 18 - 20 hours later they were killed and the uterine horns excised.

A length of 1 - 2 cm. was suspended in a 1.5 ml. bath containing oxygenated de Jalon solution with atropine sulphate (1 µg/ml) at 29-31°. The recording lever described above was used.

A sample was allowed to remain in contact with the tissue for up to 2 minutes. The cycle was repeated every six minutes.

The threshold concentration of bradykinin was commonly about 0.25 ng/ml. The dose-response curve was steep; a concentration of 1.0 on 1.5 ng/ml often elicited a maximal response.

In the majority of experiments where large numbers of fractions were being screened, the assay was of a simple bracket type. The calculations of the specific activities of the most highly purified preparations of kininogen were however based on 2 + 1 dose assays and the calculation of fiducial limits. Figure 9 shows a sample record.

Rat duodenum

The first 2 - 3 cm of the duodenum was suspended in oxygenated de Jalon at 29 - 31°. The fluid in the 2 ml. bath was changed by overflow but the flow was stopped for up to 1 minute after the addition of a sample. The frontal writing system exerted a tension of 0.5 g and magnified about ten fold.

A representative trace is shown in figure 10.

4. Specimen of 2 + 1 dose assay

A sample of purified bradykininogen (fraction 69, fig. 6) was incubated with trypsin and the released kinin was assayed on the rat uterus. The potency ratio and its fiducial limits were calculated in the way described by J.H. Gaddum (1953). Pharmacol. Rev., 5, 119.

preparation - rat uterus, de Jalon, 30°, atropine sulphate 1 µg/ml.

standard - synthetic bradykinin. (concentrations of 1.0 and 1.5 ng/ml.)

unknown - fraction 69 incubated with trypsin and diluted 1 : 600

U =69/600 S_1 =1.0ng/ml S_2 =1.5ng/ml (S=1.25ng/ml)

Results	contraction height=y(mm)					$\Sigma y'$	$\bar{y}'$
S_1	47	59	50	51	36	243	48.6
U	51	61	58	54	53	277	55.4
S_2	63	68	66	63	67	327	65.4
						Σy 847	

Calculation of log potency ratio

$\bar{y}_u = 55.4$; $\bar{y}_{s_1} = 48.6$; $\bar{y}_{s_2} = 65.4$ $(\bar{y}_s = 57)$

E = "dose difference" $= \bar{y}_{s_2} - \bar{y}_{s_1} = 16.8$

I = log. ratio of doses $= \log \frac{1.5}{1.0} = 0.176$

b = slope $= E/I = 95.5$

F = "preparation difference" $= \bar{y}_s - \bar{y}_u = 1.6$

M = log. potency ratio $= \frac{F}{b} = 0.0167$

potency ratio $= \frac{U}{S} = 1.040$

U = 1.040 x 1.25 ng/ml.

potential bradykinin in fraction 69 = 600 U = 780ng/ml.

Calculation of fiducial limits of M

$\Sigma y^2 = 48,905$

$(\Sigma y)^2 = 717,409$

$n = 15$

variance of y $= \frac{\Sigma y^2 - \frac{(\Sigma y)^2}{n}}{n-1} = 77$

variance of $\bar{y}$ $= V = \frac{77}{n} = 5.13$

variance of F $= V_F = \frac{3V}{2} = 7.7$

variance of b $= V_b = \frac{2V}{I^2} = 331$

$t\ (P = 0.05,\ n-1 = 14) = 2.14$

fiducial limits of M $= M \pm \frac{t}{b}\sqrt{V_F + V_b M^2}$

$= 0.0167 \pm 0.0625$

fiducial limits of potency ratio = 0.90 - 1.20

potential bradykinin in fraction 69 = 780 ± 120ng/ml.

5. Measurement of protein concentration

The optical density at a wavelength of 275 mμ was determined using a Unicam SP 500 Spectrophotometer. This was used as an approximate measure of protein concentration.

More accurate determinations were made by the method of Lowry, Rosenbrough, Farr and Randall (1951) which uses the Folin, Ciocalteau reagent. The reagent and sample volumes were doubled to allow the use of silica cuvettes with a 1 cm. light path. No other modifications were made.

Standard calibration curves were prepared with diluted normal human serum assuming a protein concentration of 70 mg/ml. This standard was used for the measurement of protein concentrations in the fractions from the DEAE-Sephadex columns (table III).

In later experiments with the purified kininogen eluted from the G-200 Sephadex columns, bovine serum albumin was used as the standard. A standard calibration curve was determined with each new group of measurements.

6. Measurement of specific conductivity

All determinations were made on a Philips conductivity bridge (type PR 9500) at 1,000 c.p.s.

Approximate measurements for the location of the electrolyte peak in the eluate from a column were made at room temperature using a micro-cell (constant = 0.70).

Accurate measurements were made to check the composition of the buffers used in the stepwise elution of kininogen from DEAE - Sephadex. For this purpose the samples were brought to 25 $\pm$ 0.5° in a water bath and larger cells were used (constant = 1.33).

FRACTIONAL PRECIPITATION AND EXTRACTION:

7. Fractional extraction with ammonium sulphate solutions

The tables below summarise the results of four experiments in which the purification of bradykininogen was attempted by the fractional extraction of plasma protein precipitates.

experiment	plasma volume (ml)	pH	t°
(1)	10	6	20
(2)	10	6	4
(3)	20	6	4
(4)	20	4	4

ammonium sulphate moles/l	kininogen in extract (units)			
	(1)	(2)	(3)	(4)
1.8	3	12	4	6
1.6	12	27	16	6
1.4	10	10	10	6
1.2	5	12	5	10
1.0	<2	9	<1	<4
0.8	-	-	<1	<4

8. Potassium phosphate buffers

Phosphate buffers of constant pH (5.8) but graded ionic strength were prepared from the data of Green and Hughes (1955). The composition of the buffers used in conjunction with a plasma protein dilution of 1:11 is shown below.

A = initial potassium phosphate concentration of buffer.

B = final concentration of potassium phosphate after the addition of 1 vol. plasma to 10 vol. buffer.

A moles/l	B moles/l	$\frac{K_2HPO_4}{KH_2PO_4+K_2HPO_4}$	KH_2PO_4 g/l	N.KOH ml/l
1.54	1.4	0.244	209	341
1.76	1.6	0.258	239	414
1.98	1.8	0.269	269	484
2.20	2.0	0.280	299	560
2.42	2.2	0.290	329	638
2.64	2.4	0.300	359	740
2.86	2.6	0.310	389	807

GEL FILTRATION OF PLASMA

9. G-50 Sephadex, : preparation packing and regeneration

Coarse G-50 Sephadex (water regain 5.0g/g dry gel: no 9860 C) was obtained from A.B. Pharmacia (Uppsala). It was allowed to swell in buffer for several hours. Fine particles were removed by sucking off the supernatant after the bulk of the particles had settled. When this process had been repeated several times the supernatant was clear. The slurry was then exposed to low pressures to remove dissolved air.

The delivery tube of the siliconed glass column was packed with a skein of polythene turnings and an extension tube was fitted to the top. The slurry was poured and the column packed in the way described by Flodin (1962). The upper layer was stirred to ensure a flat surface but no disc was added.

The plasma load was introduced through a 1mm. bore polythene catheter a few mm. above the top of the gel. 10ml. of plasma could be layered in this way within 5 - 10 minutes without disturbance of the gel or mixing with the buffer.

After use the column was dismantled and the process of sedimentation in buffer repeated. The gel was stored in 2M. salt at 4°.

10. Column dimensions and characteristics

The table gives the characteristics of the G-50 Sephadex columns used for the equilibration of different volumes of plasma with buffer. The excluded water volumn (Vo) was determined from the position of the optical density peak and the total water volume (Vo + Vi) from the electrolyte peak.

column length cm.	column diameter cm.	Vo ml.	Vo+Vi ml.	Vol. of plasma applied ml.	Vol. of eluate collected ml.
45	2.4	55	170	30	100
25	2.4	35	95	20	80
15	2.4	20	58	10	50
10	1.8	8	23	5	18

ANION EXCHANGE CHROMATOGRAPHY

11. Sodium phosphate buffers

The composition of the buffers used for the fractionation of plasma on DEAE-Sephadex is shown in Table VIII

12. DEAE- Sephadex : preparation, packing and regeneration.

DEAE- Sephadex, A-50 medium (water regain = 5.0 g/g, anion capacity 3.5 ± 0.5 mE/g) was obtained from Pharmacia. Samples from batches 7874 and 1602 were used.

The gel was swollen, freed from fine particles and freed from dissolved air in the same way as the G-50 Sephadex. The slurry was poured and the column packed. Buffer was passed until the pH of the eluate was identical with that of the buffer applied.

After use the gel was transferred to a no. 54 whatman filter paper in a Büchner funnel. It was stirred with 2 volumes of 0.5 N. NaOH and then washed with distilled water under suction. After stirring with 2 volumes 0.5 N.HCl, the gel was washed with several litres of distilled water followed by buffer. This process appeared to produce several fine particles but the subsequent behaviour of the rest of the gel was not altered.

Table VIII

Composition of sodium phosphate buffers

Na Cl		$NaH_2PO_4\ 2H_2O$	$Na_2HPO_4\ 12H_2O$		Specific conductivity*
moles/l	g/l	g/l	g/l	pH	m.mho/cm
0.10	5.85	0.50	6.02	7.35	12.2
0.13	7.60	0.50	6.02	7.30	14.8
0.16	9.35	0.50	6.02	7.25	17.2
0.18	10.52	0.50	6.02	7.20	18.8

* 25°

Appendix 13

The fractionation of plasma on DEAE- Sephadex : Summary of 8 experiments

plasma volume (ml)	G-50 Sephadex column length (cm)	DEAE-Sephadex column length (cm)	Free kinin (µg Bk)	Bradykininogen (units/fraction)			total kininogen (units/ml plasma)
			A	B	C	D	
5	10	7.5	1.2	7.5	15	1.5	4.8
15	12.5	7.5	3	20	30	4.5	3.6
30	25	7.5	-	-	30	-	1.0
10	25	15	3	3	15	6	2.4
10	15	15	1.5	3	24	4	3.1
10	15	15	1.5	1.5	24	4	3.0
20	25	15	-	-	30	-	1.5
20	25	15	-	-	40	-	2.0

Appendix 14

The fractionation of 10 ml. plasma on DEAE- Sephadex A-50 (3.5 x 15 cm) : Summary of 4 experiments

a = free kinin (μg Bk/fraction) b = free kinin + kinin released by trypsin (μg Bk/fraction)

Fraction		Bradykinin, μg/fraction = y (1)	(2)	(3)	(4)	Σy	$\bar{y}$	$\sum^{x}(\bar{y}-y)^2$	S.E. $\frac{x}{n-1}$	$\bar{y} \pm$ S.E.
A	a	1.6	3.0	2.0	1.5	8.1	2.0	1.41	0.68	2.0 ± 0.7
	b	1.1	3.0	1.5	1.5	7.1	1.8	2.11	0.83	1.8 ± 0.8
B	a	0.6	1.0	1.6	1.6					
	b	15.5	6.0	6.0	6.0	35.5	8.4	66.8	4.7	8.4 ± 4.7
C	a	0.6	1.0	1.0	1.0					
	b	38	60	45	45	188	47	258	9.3	47 ± 9.3
D	a	0.6	1.0	1.0	0.5					
	b	6.2	3.0	4.0	4.0	17.2	4.3	6.1	1.4	4.3 ± 1.4

GEL FILTRATION OF FRACTION C

15. G-200 Sephadex : preparation, packing and

G-200 Sephadex (water regain 20.0g/g dry gel: lot no. 64) was obtained from A.B. Pharmacia, Uppsala.

The particles were allowed to swell for at least 48 hours in distilled water containing sodium chloride (about 0.5M). Particular care was taken during the sedimentation in order to reduce the number of fine particles to a minimum.

The columns were packed on top of a support which consisted of cotton wool, 2mm. glass beads and coarse G-50 Sephadex. The top of the column was protected by a filter disc and a 1 cm. layer of coarse G-50 Sephadex. These were necessary in order to prevent loads of high specific gravity from displacing loosely packed G-200 Sephadex.

Samples were loaded in the way described for plasma.

Regeneration was carried out by reversing the flow of buffer for several hours after removing the G-50 Sephadex from the top of the column.

16. Reference proteins

The following proteins were compared with human kininogen on G-200 Sephadex.

twice crystallised ovalbumin (salt-free) L. Light and Co., Colnbrook, England.

bovine serum albumin (fraction V) batch CH 3170 Armour Pharmaceutical Co. Ltd., Eastbourne, England.

rabbit gamma globulin (fraction II) lot 29. Pentex Incorporated, Kankakee, Illinois, U.S.A.

PROPERTIES OF PURIFIED KININOGEN

17. Starch gel electrophoresis

materials

partially hydrolysed starch (Smithies). British Drug Houses, Ltd., Poole, England.

potato starch, B.D.H., Ltd.

low melting point paraffin (30 G. white vaseline, 25ml. liquid paraffin, 25 ml. petrol ether).

amido black in water : methanol : acetic acid (5 : 5 : 1).

methods

The method (Cruft and Leaver, 1961) was a small scale modification of the original. (Smithies, 1955). Starch gel was prepared as described by Smithies and poured onto thin glass plates. The gel was covered with a polythene sheet and rolled flat.

The protein samples were mixed into a paste with starch grains and placed in troughs (0.1 x 1.0 cm) cut in the gel.

Evaporation was prevented by a coat of low melting point paraffin. After passing 2 m.A. for 10 hours, the slides were washed in petrol ether and stained with amido black.

When trypsin incubation and biological assay were performed the unstained gel was divided by a vertical, longitudinal cut. Before the two halves were separated a series of transverse slits were made at $\frac{1}{2}$ cm. intervals across the longitudinal cut. In the half for bioassay the strips were separated. After staining the other half it was possible, despite shrinkage to relate the stained zones to the cut strips.

18. Immunoelectrophoresis

materials

Agar (Ionagar no. 2. Code no. L 12. batch 32, Oxo, Ltd., London)

Veronal acetate buffer 0.05 M. pH 8.6.

Horse antihuman serum. Institut Pasteur, Paris.

lot 13461

lot 223.

Horse antihuman serum. Wellcome, London.

lot 4505

lot 4506

methods

The method of Scheidegger (1955) was followed. Protein samples were dissolved in 0.05M veronal acetate buffer, pH 8.6 and placed in circular wells cut in 1% agar gel on microscope slides. A current of 5 m.A. was passed for 1¼ hours and antiserum was added to the troughs. The slides were then observed during a period of 72 hours at 4° and photographed at suitable stages.

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